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Clinical and Experimental

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VOL. 45 FEBRUARY, 1955 NO. 2

CLINICAL AND EXPERIMENTAL

RADIOIRON STUDIES IN HEMOCHROMATOSIS

THE EFFECTS OF REPEATED PHLEBOTOMIES

T. H. BOTHWELL, M.D., M.R.C.P.,* B. C. ELLIS, M.B., B.CH.,** H. VAN W. VAN DOORN-WITTKAMPF, M.D., AND O. L. ABRAHAMS, B.SC.**

JOHANNESBURG, SOUTH AFRICA

RECENTLY Huff and co-workers[14] have described a method for estimating the plasma and red cell iron turnovers in the body. The present investigation was undertaken to determine such turnovers in hemochromatosis. The results were compared with those obtained in a control group of healthy subjects and in one case of malnutritional cytosiderosis.[10] In addition, the effects of repeated phlebotomies on iron metabolism in hemochromatosis were investigated. At the time these studies were done the radioiron used was of a lower specific activity than is now available and this made the analysis of data more difficult. In spite of this qualification the results obtained were considered interesting and worthy of publication.

MATERIAL AND METHODS

Clinical Material.—Two male and two female cases of hemochromatosis were studied. In each the diagnosis was confirmed by liver aspiration biopsy. Of the females Case 15, aged 55 years, was postmenopausal and the other (Case 14), aged 23 years, had amenorrhea of 4 years' duration. Skin pigmentation and cirrhosis of the liver were present in all, none were anemic, and Cases 12, 13, and 15 had diabetes.

The control group consisted of 8 healthy men and 3 healthy women, two of whom were postmenopausal (Cases 9 and 10). Their ages ranged from 20 to 65 years.

The one case of malnutritional cytosiderosis studied was a South African Negro, aged 64 years, who presented with diabetes. Histologic examination of a liver biopsy showed heavy hepatocellular and portal iron deposits with portal fibrosis and bile duct hyperplasia.

Preparation of Radioiron.—The methods were similar to those used previously.[1] Radioiron derived from the Szilard-Chalmers reaction was supplied by the Atomic Energy Research Establishment (Harwell, England) as Fe^{59} of specific activity varying from 10 to 30 μg per microcurie. The iron was in the form of a ferric chloride stock solution and tracer doses made

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up from this had activities varying from 4 to 10 μc on the day of administration. Each dose was autoclaved in rubber-capped bottles at a pressure of 20 pounds p.s.i. for 20 minutes. On the day of study 15 to 20 ml. of normal plasma was rapidly added to this solution and allowed to stand for about 10 minutes to enable the iron to attach itself to the β_1 globulin. In earlier studies the stock doses of about 3 ml. of ferric chloride solution were buffered to pH 6 with 3.8 per cent sodium citrate. However, it was found early that the solution was effectively buffered merely by the addition of the plasma, and this method was used in all later studies. The amount of elemental iron administered varied from 50 to 300 μg (mean 135 μg).

Administration of Radioiron.—All subjects were basal at the time of study. About 20 ml. of the plasma-iron dose was injected intravenously, the weight administered being determined by difference. An aliquot of 1 to 2 ml. of this solution was retained for subsequent analysis in order to determine the exact dose of radioiron and elemental iron given. Thereafter blood samples of about 3 ml. were taken serially into weighed tubes each containing 6 mg. of mixed oxalate. The tubes were again weighed and the weight of each blood sample determined by difference. In the initial 6 hours samples were taken at 30-minute intervals, but when a rapid plasma-iron turnover was expected, more frequent sampling was carried out within the first 3 hours. The red cell iron uptake was determined in most subjects by taking daily blood samples between the seventh and fourteenth days. In the earlier studies the plasma from each blood sample was pipetted off and added to 3 red cell washings. Separate analysis for radioactivity could then be made on plasma and red cells. However, it became apparent that the amount of radioiron that could be recovered from red cells in the early samples and from the plasma in the later samples was so low as to be of no account. In subsequent studies, therefore, no separation was done except in patients to whom a previous dose of radioiron had been given. In addition, in such cases basal blood samples were taken for radioiron analysis prior to giving the next radioiron dose. It was assumed that any residual radioactivity in the blood from previous tests was constant throughout the period of the subsequent study and corrections for red cell uptakes were made on this assumption.

In the hemochromatotic subjects repeat radioiron studies were done after the removal of various quantities of blood (Fig. 1). Phlebotomies (300 to 800 ml.) were performed at 3- to 10-day intervals and frequent hemoglobin, serum iron, and iron-binding capacity values were estimated. In Case 12 repeat radioiron studies were done after the removal of 5,190 ml. and again after a total of 13,110 ml. had been taken off. Case 13 had a repeat study performed after 1,800 ml. blood had been removed, and 3,650 ml. was taken off prior to the second study in Case 14. In Case 15 radioiron tests were done after 5,060 ml. and 9,610 ml. blood, respectively, had been removed. The time intervals between the commencement of the phlebotomies and the subsequent radioiron studies in each case can be seen in Fig. 1. In two normal subjects (4 and 11) repeat plasma iron turnovers were performed 48 hours after the initial ones.

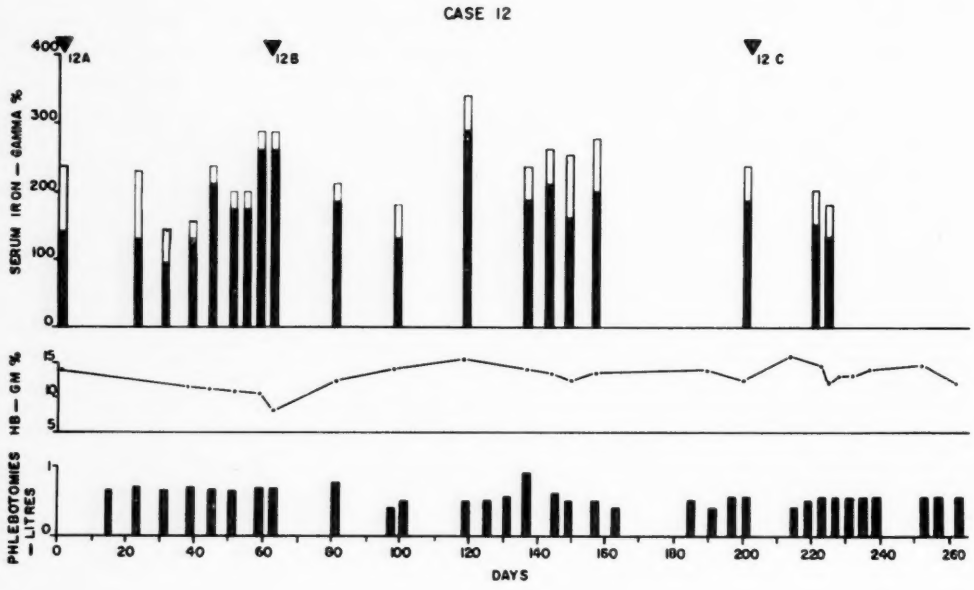
Blood Measurements.—Radioiron analyses on all blood samples were performed by electroplating them according to a slightly modified technique of Vosburgh, Flexner, and Cowie.[22] The samples were weighed, dry-ashed, chemically processed, and plated on copper discs in electroplating cells. These were then counted with a G.E.C. Counter (type E.H.M. 2-mica window, with a window thickness of 1.9 mg. per cm.^{2}) and the counts were compared with those due to plated standards prepared by the same technique from aliquots of the stock solution from which the patient's dose was derived. Activities of plated samples could thus be given in terms of the administered tracer dose. The specific gravities of the plasma and whole blood were determined by the copper sulfate method.[18] From these data the weights of plasma and red cells in each sample were converted into volumetric measurements. Serum iron values were determined in duplicate by the method of Kitzes and associates.[17] Iron-binding capacities were estimated by the method of Rath and Finch[19] modified for use with the Evelyn colorimeter. Total hemoglobin estimations were performed by a cyanhematin method[16] adapted for use with the Evelyn colorimeter. Blood volume determinations were made by a T1824-hematocrit method.[6] The values for red cell mass were reduced by 15 per cent since it has frequently been shown that such an overestimation occurs with the dye method.[20]

Analysis of Data.—Recently it has been shown that when a tracer dose of radioiron of high specific activity is added to plasma and injected intravenously it disappears exponentially, thus reflecting the normal course of the transport iron through the plasma iron pool. Thereafter, some of the radioiron reappears in the hemoglobin of the red cells. With a knowledge of the plasma iron level and of the plasma and red cell volumes it is then possible to measure both the plasma and red cell iron turnovers.[14] The present data were analyzed according to these methods. However, it was found that in four control subjects and in the majority of cases with hemochromatosis the disappearance of radioiron after injection was not exponential from 100 per cent concentration at zero time. In such cases there was a rapid loss in the first few minutes which accounted for as much as 65 per cent of the injected dose in one test (Table I). Thereafter, however, the disappearance was exponential over the next few hours, suggesting that the radioiron which had initially passed into the extravascular spaces did not re-enter the plasma in significant quantities during this period. It was, therefore, considered valid to use this slow component in the calculation of plasma iron turnovers. Huff and co-workers[14] encountered similar difficulties in cases with refractory anemia and also used this slow exponential component in the calculation of their results. The subsequent course of the radioiron which disappeared early from the circulation is uncertain. However, there is experimental evidence that at least some of this unattached ferric chloride is stored in the liver.[7] In the present study it was also found that some of this radioiron was later fed back into the circulation as the percentage of the injected dose appearing in the red cell mass of certain cases of hemochromatosis was greater than the percentage initially attached to the β_1 globulin of the plasma (Table I). The actual quantity of radioiron redelivered to the circulation from the extravascular compartment in such instances must depend on several factors, viz., the size of the iron stores, the degree of equilibrium between radioiron and nonradioactive iron in these stores, and the percentage being fed back from the extracellular spaces. As there is no way of measuring the relative importance of these factors or the proportions of radioiron re-entering the circulation from these various sites, it would seem to be invalid to calculate the red cell iron turnover (percentage radioiron in red cell mass × plasma iron turnover) in such cases. Therefore red cell iron turnovers were calculated only in studies where the initial disappearance of radioiron was exponential from 100 per cent concentration at zero time. In the others the red cell iron utilization was expressed as a percentage of the administered dose.

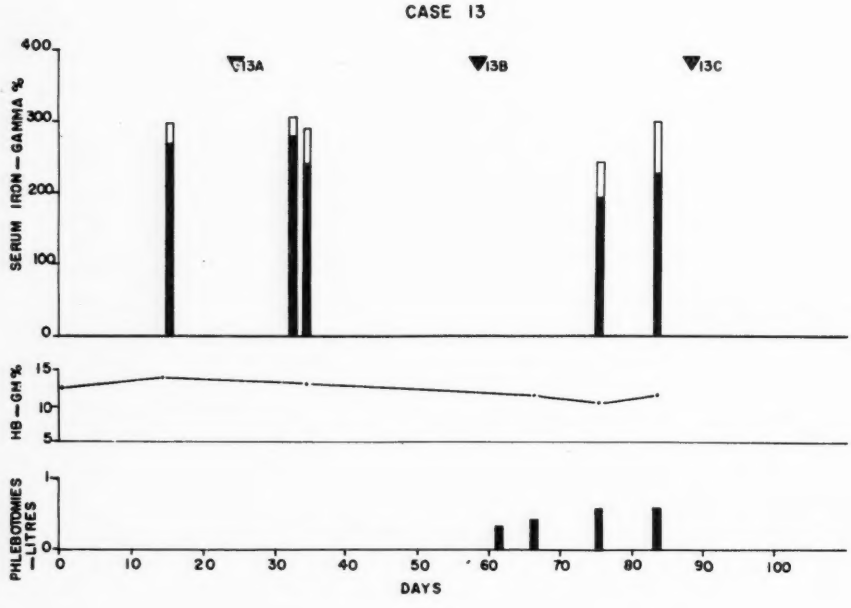
The incomplete attachment of radioiron to the plasma globulin in some cases seemed to depend on two factors. First, the quantity of iron which was injected was relatively large, the average amount being 135 μg (range 30 to 300 μg). Although the doses were prepared with normal human plasma (collected with precautions against iron contamination) there was still insufficient unsaturated β_1 globulin present to ensure that such amounts of iron would all be bound. However, it might be expected that at least some of this unbound iron would become attached intravascularly, especially as these doses were injected over a period of about 5 minutes. The second factor was probably related to the plasma iron-binding capacity which was reduced in the hemochromatotic subjects. However, in spite of the incomplete binding of the radioiron the doses could be regarded as true tracers in so far as the bound fractions did not alter the plasma iron component significantly. In these cases the average amount of radioiron which was attached to the β_1 globulin of the plasma was 80 μg (range 20 to 170 μg) and the addition of such amounts of iron could not produce any measurable change in the plasma iron level.

RESULTS

Plasma Iron Turnover.—In 13 studies on 11 control subjects the mean plasma iron turnover was 30 mg. per day (range 17 to 40 mg. per day) and 0.46 mg. per kilogram per day (range 0.29 to 0.62 mg. per kilogram per day). The turnover in milligrams per day was higher in men (9 studies) as compared with women (4 studies), but this difference was not apparent when the results

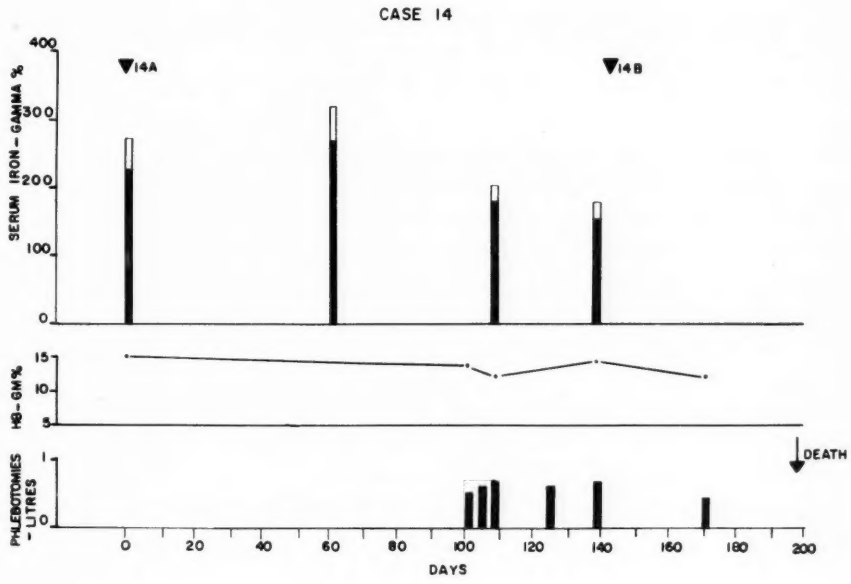


A.



B.

Fig. 1, *A* and *B*. *(For legend see opposite page.)*



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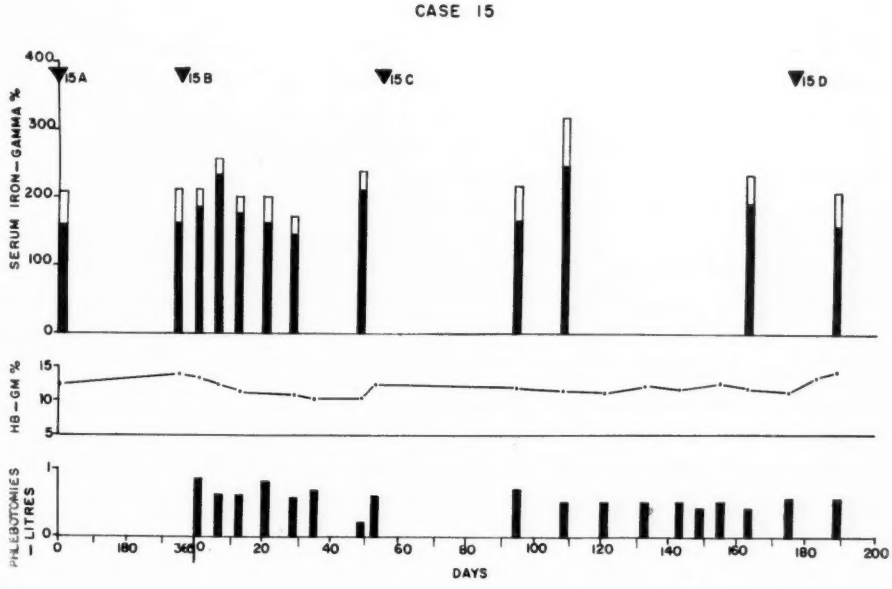

D.

Fig. 1.—*A*, *B*, *C*, and *D*. The effects of repeated phlebotomies on the serum iron, iron-binding capacity, and hemoglobin levels in four cases of hemochromatosis. The times are shown at which the radioiron studies reported in Table I were done. The open areas of the serum iron columns represent the iron-binding capacity.

TABLE I

CASE NO.	SEX	AGE (YR.)	INJECTED RADIOIRON BOUND TO β_1 GLOBULIN OF PLASMA (%)†	TIME TAKEN TO REACH ½-CONCENTRATION (HR.)‡	PLASMA IRON TURNOVER (MG./DAY)	PLASMA IRON TURNOVER (MG./KG./DAY)	PLASMA IRON REMOVED (FRACTION PER HR.)	RED CELL IRON TURNOVER (MG./DAY)	RED CELL IRON TURNOVER (MG./KG./DAY)	RED CELL IRON RENEWED (FRACTION PER DAY)	RED CELL IRON UTILIZATION (%)
Normal											
1	M	45	100	1.2	28	0.40	0.59	25	0.36	0.015	89
2	M	20	95	1.6	38	0.54	0.42	—	—	—	85
3	M	20	100	2.1	33	0.41	0.35	33	0.41	0.013	100
4 A	M	46	100	1.1	40	0.57	0.63	*	*	*	*
4 B	M	46	100	1.0	39	0.56	0.61	*	*	*	*
5	M	22	85	1.6	35	0.56	0.41	—	—	—	82
6	M	22	100	2.0	24	0.29	0.34	20	0.24	0.006	83
7	M	23	100	2.4	24	0.38	0.29	21	0.33	0.009	88
8	M	24	90	1.7	40	0.56	0.40	—	—	—	82
9	F	52	100	1.6	17	0.32	0.44	17	0.32	0.012	100
10	F	65	100	1.0	29	0.62	0.66	29	0.62	0.020	100
11 A	F	37	45	1.8	22	0.34	0.40	*	*	*	*
11 B	F	37	100	1.6	25	0.38	0.45	*	*	*	*
Mean				1.6	30	0.46	0.46	24	0.38	0.013	90
Hemochromatosis—before phlebotomies											
12 A	M	63	100	1.2	42	0.75	0.58	21	0.38	0.016	50
13 A	M	60	100	2.0	46	1.01	0.35	25	0.55	0.019	54
13 B	M	60	35	1.9	38	0.78	0.36	*	*	*	*
14 A	F	23	55	1.7	56	0.83	0.47	—	—	—	29
15 A	F	55	60	1.6	42	0.87	0.45	—	—	—	20
15 B	F	55	40	1.9	36	0.59	0.38	—	—	—	43
Mean				1.7	43	0.80	0.43	23	0.46	0.018	39
Hemochromatosis—after phlebotomies											
12 B	M	63	100	0.6	106	1.91	1.08	76	1.36	0.076	72
12 C	M	63	50	0.7	101	1.67	0.98	—	—	—	67
13 C	M	60	40	0.7	105	2.20	0.94	—	—	—	51
14 B	F	23	40	0.7	72	1.39	0.99	—	—	—	42
15 C	F	55	55	1.1	73	1.05	0.60	—	—	—	32
15 D	F	55	45	0.9	68	1.07	0.74	—	—	—	58
Mean				0.8	87	1.55	0.89				54
Malnutritional cytosiderosis											
16	M	64	50	2.4	34	0.46	0.29	—	—	—	34

*Not done.

-Not calculated as the exponential plasma iron disappearance slopes did not extrapolate to 100 per cent at zero time (see text).

†These quantities represent the extrapolated concentration of the exponential plasma iron disappearance slopes at zero time and are assumed to be equivalent to the amount of injected radioiron bound to the β_1 globulin of the plasma.

‡In those cases where not all the injected radioiron was bound to the plasma β_1 globulin the extrapolated values at zero time were taken as 100 per cent and the ½-concentration times corrected accordingly (see text).

were expressed in milligrams per kilogram per day. In two subjects (Cases 4 and 11) repeat studies were performed 48 hours after the initial ones. No significant change in the plasma iron turnover was noted in either subject. The fraction of plasma iron removed per hour in normal subjects was 0.46 (range 0.29 to 0.66).

In 6 studies on 4 cases of hemochromatosis the mean plasma iron turnover was 43 mg. per day (range 36 to 56 mg. per day). Expressed in terms of body weight the mean was 0.80 mg. per kilogram per day (range 0.59 to 1.01 mg. per kilogram per day). The mean weight of the hemochromatotic subjects (before phlebotomies) was 54 kg. and that of the control group was 65 kg. This difference would account for the apparent discrepancy between these two groups of the values expressed in terms of body weight. The fraction of plasma iron removed per hour was 0.43 (range 0.35 to 0.58). Repeat basal studies performed in 2 subjects (Cases 13 and 15) gave results for plasma iron turnover in milligrams per day which approximated fairly closely those found previously. In one (Case 15), however, there was a marked reduction in the plasma iron turnover expressed in milligrams per kilogram per day from 0.87 in the first to 0.59 in the second study. This was due to a marked gain in weight (from 48 to 61 kg.) in the four-month period between the two investigations. After repeated phlebotomies the mean plasma iron turnover was raised to 87 mg. per day (range 68 to 106 mg. per day) and 1.55 mg. per kilogram per day (range 1.05 to 2.20 mg. per kilogram per day). The fraction of plasma iron removed per hour was increased to 0.89 (range 0.60 to 1.08). In Cases 12 and 15 further studies were done after the removal of additional volumes of 7,120 and 4,550 ml. blood, respectively. The results for plasma iron turnover closely approximated those obtained previously (101 mg. per day as compared with 106 mg. per day and 68 mg. per day as compared with 73 mg. per day). The one case of malnutritional cytosiderosis studied had a plasma iron turnover which was within the normal range (34 mg. per day and 0.46 mg. per kilogram per day).

Plasma Iron Disappearance Rates.—The plasma iron disappearance rates are given in Table I. In those cases where the exponential component did not extrapolate through 100 per cent at zero time, its observed extrapolated concentration was regarded as 100 per cent and the times taken to reach ½-concentration were calculated. Although this correction was not necessary in order to calculate the turnover values it was done so that the ½-concentration times could be compared. In the control group the mean time was 1.6 hr. (range 1.0 to 2.4 hr.), and in the cases with hemochromatosis it was 1.7 hr. (range 1.2 to 2.0 hr.), which was reduced to a mean of 0.8 hr. (range 0.6 to 1.1 hr.) after repeated phlebotomies. In the case of malnutritional cytosiderosis the time was 2.4 hr.

Red Cell Iron Turnover and Percentage Red Cell Utilization.—The red cell iron turnover in 6 control subjects was 24 mg. per day (range 17 to 33 mg. per day) and 0.38 mg. per kilogram per day (range 0.24 to 0.62 mg. per kilogram per day), while the fraction of red cells renewed per day was 0.013 (range 0.006 to 0.020). In only two subjects with hemochromatosis (Cases 12 and 13)

was it possible to calculate the red cell iron turnover. Results of 21 and 25 mg. per day and 0.38 and 0.55 mg. per kilogram per day were recorded with red cell renewal fractions of 0.016 and 0.019, respectively. In Case 12 further results were obtained after repeated phlebotomies. It was found that the red cell iron turnover had been raised to 76 mg. per day and 1.36 mg. per kilogram per day and the fraction of red cells renewed to 0.076 per day.

The mean red cell utilization of the injected dose of radioiron was 90 per cent (range 82 to 100 per cent) in 9 control subjects as compared with a mean of 39 per cent (range 20 to 54 per cent) in 5 studies on 4 cases with hemochromatosis (Fig. 3). This figure was raised to a mean of 54 per cent (range 32 to 72 per cent) in 6 studies done after repeated phlebotomies on 4 subjects with hemochromatosis. In the case of malnutritional cytosiderosis the red cell utilization of injected radioiron was 34 per cent.

DISCUSSION

It has been shown that iron derived from gut absorption accounts for less than 1 per cent of the plasma iron turnover in normal subjects.[14] However, iron absorption is so high in at least some cases of hemochromatosis[1, 3, 4] that an associated increase in the turnover of iron in the plasma might be expected. In addition, there is a continual internal redistribution of iron in subjects with raised iron stores.[7] As this transfer must occur via the plasma iron compartment it should also lead to a more rapid turnover of iron in the plasma. The results of the present investigation showed that there was, in fact, a significant increase in the plasma iron turnover expressed in milligrams per kilogram per day in hemochromatosis as compared with a group of control subjects ($P < 0.01$ as calculated by the t test) (Table I and Fig. 2). Previously we have demonstrated that there was a very great absorption of iron from the gut in two young subjects with hemochromatosis, and it was suggested that the metabolic defect was more severe and accounted for the poor prognosis of such cases.[4] It is of interest, therefore, that the highest turnover of 56 mg. per day was recorded in the only young subject with hemachromatosis investigated. Iron absorption studies done previously on this case had shown a retention of 72 per cent of an oral dose of 20 mg. of iron.[4] The increased plasma iron turnover in hemochromatosis seems to be a function of the large plasma iron pool as the rate of removal of iron from the plasma was normal (Table I). In only two of these subjects was it possible to calculate the red cell iron turnover and in both it was well within the normal range. This finding might have been expected as there is usually no anemia in hemochromatosis[21] and no evidence of a shortened red cell life. It has been shown previously that the percentage red cell utilization of a small dose of injected radioiron is low in hemochromatosis as compared with normal subjects.[8] This finding was confirmed in the present study (Fig. 3). It merely reflects, however, the high level of iron stores in the disease and does not indicate diminished red cell production.

Only one case of malnutritional cytosiderosis was investigated. This condition has been found to be present in a large percentage of South African Negro pellagrins.[9] The histologic features in the livers in some of these cases

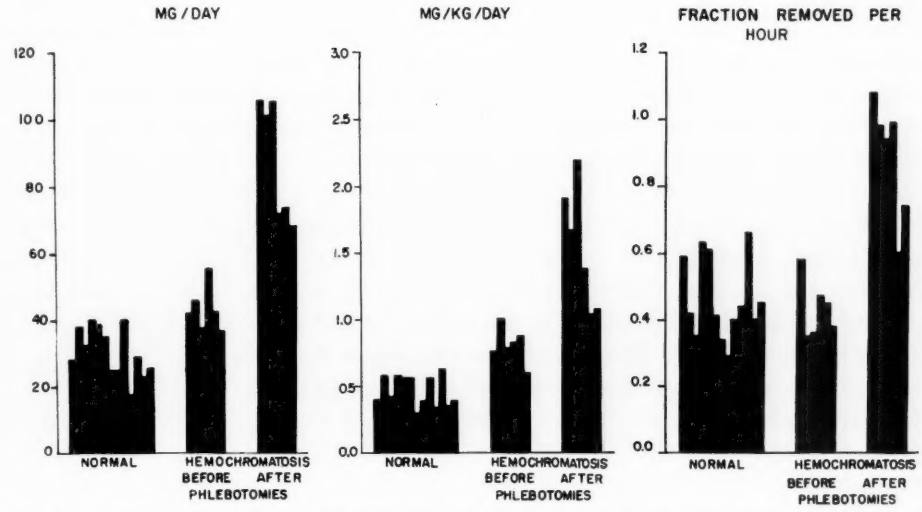

Fig. 2.—Plasma iron turnover values in normal subjects and in patients with hemochromatosis before and after repeated phlebotomies.

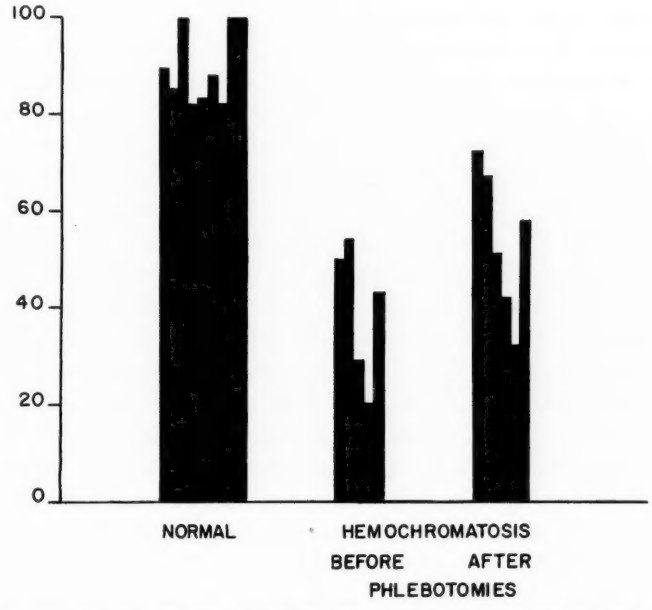

Fig. 3.—The percentage red cell utilization of an intravenous dose of radioiron in normal subjects and in patients with hemochromatosis before and after repeated phlebotomies.

were indistinguishable from those seen in hemochromatosis, and it was concluded that chronic malnutrition leads to a derangement in intracellular metabolism which interferes with the normal "mucosal block"[12] and so allows an abnormally large absorption of iron from the gut.[10] In previous oral radioiron studies on 5 subjects with malnutritional cytosiderosis, absorption from the gut was shown to be very low in 4 subjects.[3] However, in the remaining one a significant blood uptake was found and there was evidence of deposition of radioiron in the liver. It was decided to reinvestigate this last subject in the present study. Although the serum iron level was high (197 μg per cent) and the plasma volume normal, the plasma iron turnover was found to be lower than in any of the subjects with hemochromatosis and was well within the normal range. This was accounted for by the slow plasma iron disappearance rate (Table I).

There was a wide individual variation in the plasma and red cell iron turnovers in normal subjects. The plasma iron turnover in milligrams per day was significantly higher in men than in women ($P < 0.05$), but this difference was not apparent when the turnover was related to body weight as milligrams per kilogram per day ($P < 0.7$). However, the groups were small and the age distributions not strictly comparable so that no valid conclusions could be drawn from these figures. The mean results for plasma iron turnover and for red cell iron turnover, where this could be calculated, approximated fairly closely those reported by Huff and co-workers[14] in normal subjects. They were slightly higher, however, but the reason for this may be related in some way to a different physiologic pattern at the Johannesburg altitude (5,700 feet). In a later paper, however, these workers reported on findings in a group of normal students in Lima.[15] In this second study they recorded a mean plasma iron turnover of 57 mg. per day and a red cell iron turnover of 47.3 mg. per day as compared with 30 mg. per day and 24 mg. per day, respectively, in the present series. On analyzing their data it seemed that the major factor producing this difference was the high serum iron level in Lima (a mean of 180 μg per cent as compared with a mean of 109 μg per cent in the present study). Huff and co-workers[15] suggested that the high serum values might have been due to the delay between the collection of blood samples and their subsequent analysis. There are no other available data for iron turnovers in normal subjects. However, Wasserman and co-workers[23] have studied the rate of disappearance of plasma iron in such a group. Calculations from their data show the mean time taken to reach ½-concentration to have been 1.5 hr., a figure which agrees closely with the one of 1.6 hr. found in the present study.

It has been suggested that repeated phlebotomies might form a rational form of therapy in hemochromatosis[11] and several investigators have subsequently treated cases in this way.[2, 5, 13] It has been found that these subjects are unique in their ability to maintain their hemoglobin levels after the repeated removal of large quantities of blood. One reported case tolerated the removal of as much as 50 L. of blood in 1 year and showed evidence of both subjective and objective improvement.[5] In the present study 4 patients with hemochromatosis were treated by this method and the serial changes in hemoglobin and serum iron levels can be seen in Fig. 1. One case (14) died relatively soon after the commencement of this therapy, but it was felt that this

was not causally related as she had already been in congestive cardiac failure several times prior to the commencement of phlebotomies. The others tolerated repeated phlebotomies well, but so far it is not possible to say whether the ultimate prognosis has been altered in any way.

The turnover of iron in hemochromatosis was studied in each patient after repeated phlebotomies. There was a striking rise in the plasma iron turnover. Four studies were done relatively soon after the commencement of treatment. In two (Cases 12 and 15) repeat studies were performed after the removal of further large quantities of blood (Fig. 1) and it was found that the plasma iron turnover was maintained at a constant figure for the individual. However, the capacity to mobilize iron stores for increased blood formation seemed to be different in each subject and this was reflected by the different frequencies with which phlebotomies could be performed on them. The increased plasma iron turnover following repeated phlebotomies in hemochromatosis was found to be due to an increased disappearance rate of iron from the plasma (Table I). It was possible to measure the red cell iron turnover in only one subject after repeated phlebotomies. It was found that it had risen from 21 mg. per day to 76 mg. per day. The plasma iron turnover at this time was 106 mg. per day. It is, therefore, evident that although the patient was being maintained in a negative iron balance by repeated phlebotomies, only about 75 per cent of the tagged plasma iron was being utilized for hemoglobin formation. Presumably the remainder was being deposited in various body stores. It is interesting to speculate as to whether this finding is an indication of an increased avidity of the tissues for iron as originally postulated by Sheldon.[21] If this is true then the increased absorption from the gut would seem to be a secondary factor and would not represent the primary defect in the disease.

SUMMARY AND CONCLUSIONS

Radioiron turnover studies were carried out on 4 patients with hemochromatosis, 1 patient with malnutritional cytosiderosis, and 11 normal subjects. A total of 26 studies were done. In addition, the effects of repeated phlebotomies in hemochromatosis were investigated.

The mean plasma iron turnover was found to be significantly raised in hemochromatosis, while the red cell iron turnover was normal in the two patients in whom it could be measured. The plasma iron turnover in the one case of malnutritional cytosiderosis fell within the normal range. The percentage of an administered dose of radioiron utilized for hemoglobin formation was reduced in hemochromatosis and in the subject with malnutritional cytosiderosis.

Repeated phlebotomies were well tolerated in hemochromatosis. They caused a marked and sustained increase in the plasma iron turnover. The degree to which the turnover could be raised varied in each subject but repeat studies in two cases seemed to show that it was constant for the individual. The red cell iron turnover was increased after repeated phlebotomies in the one subject in whom it was possible to measure it and the percentage red cell utilization of the injected dose of radioiron was raised although it was still lower than normal.

We wish to express our indebtednes to Professor G. A. Elliott, Head of the Department of Medicine, University of the Witwatersrand, for his generous assistance and encouragement throughout the study. In addition we are grateful to Dr. H. B. Stein, Acting Head of the Department of Clinical Pathology, for his co-operation and helpful advice and to Dr. B. van Lingen for criticizing the manuscript. Finally we wish to thank Dr. J. Stafford for facilities in the Biophysics Laboratories of the Council for Scientific and Industrial Research, Pretoria, and Mr. J. E. Kerrich, Department of Mathematics, University of the Witwatersrand, for advice on the statistical analysis.

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THE EFFECT OF A HEAD-UP TILTED POSITION AND AMBULATION ON RENAL HEMODYNAMICS AND WATER AND ELECTROLYTE EXCRETION IN PATIENTS WITH HYPERTENSION, WITH AND WITHOUT RENAL DAMAGE

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INTRODUCTION

THE changes observed in renal hemodynamics and the renal excretion of water and electrolytes due to the orthostatic position in normal subjects have been studied.[1-9] These observations indicate that changing from the supine to the upright position produces a state of stress resulting in reflex vasoconstriction in which the kidney participates. The current study was undertaken in patients with hypertension, with and without renal damage, in an effort to compare the effect of tilting and ambulation on water and electrolyte excretion, and the renal hemodynamic responses of these patients with those of the normal subject with normal kidneys.

METHODS

Renal function was determined by standard techniques in twenty-seven male patients. Glomerular filtration rate (GFR) (inulin clearance), renal plasma flow (RPF) (para-aminohippurate clearance), maximum tubular excretory capacity (TmPAH), blood pressure, and the rates of excretion of water, sodium, and potassium were observed. The techniques for determining renal function and the methods of analysis have been described previously.[10]

Observations on the effects of tilting were made on twenty patients with essential hypertension. None of the patients gave a history of primary renal disease. In so far as could be determined by history, any renal damage that existed was secondary to the hypertension. The control observations consisted of two consecutive 10-minute periods in recumbency. These were followed by two consecutive 10-minute periods with the patient in the 60-degree head-up passively tilted position. The patient was then returned to the recumbent position and primed with PAH. The infusion was changed to a concentrated PAH solution in order to maintain the blood level of PAH between 55 and 90 mg. per cent for the TmPAH determinations. After a 30-minute equilibration period, two periods in the recumbent and two in the 60-degree tilted position were collected in order to determine simultaneous GFR and TmPAH in thirteen patients.

The effect of ambulation on renal hemodynamics was observed in seven patients with essential hypertension. Measurements were made of glomerular filtration rate, renal plasma flow, and maximum tubular excretory capacity (TmPAH). The rates of urine formation,

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sodium and potassium excretion, and the changes in blood pressure were observed. The control determinations of glomerular filtration rate (GFR) and renal plasma flow (RPF) consisted of three consecutive 10-minute periods in recumbency, followed by three periods in the ambulatory state. During the ambulatory studies the patients pushed the infusion apparatus (mercury pump, tubing, clamps, etc.) mounted on a small table equipped with wheels. The infusion apparatus was connected to an indwelling needle in the antecubital vein so that a constant intravenous infusion of PAH and inulin was maintained at all times. Also, a manifold mounted on the table was connected to the antecubital vein of the opposite arm for obtaining repeated blood samples in the ambulatory state. The priming dose of PAH for determining TmPAH was then administered. Following a 60-minute equilibration and rest period in the recumbent position, three control determinations of GFR and TmPAH were performed. These were followed by three periods in the ambulatory state.

TABLE I. EFFECT OF TILT ON RENAL HEMODYNAMICS

PA-	MBP			GFR (IN.)			RENAL PLASMA FLOW			RENAL BLOOD FLOW			RVR		
TIENT	C	T	%C	C	T	%C	C	T	%C	C	T	%C	C	T	%C
1	156	151	97	9	9	100	80	72	90	110	99	90	1.418	1.525	108
2	164	147	90	27	24	89	131	97	74	226	194	86	0.726	0.758	104
3	156	163	104	37	39	105	214	221	103	340	345	101	0.459	0.472	103
4	147	145	99	46	11	24	285	125	44	365	169	46	0.403	0.858	213
5	117	110	94	45	19	42	376	247	66	530	358	68	0.221	0.307	139
Sub.Av.	148	143	97	33	20	72	217	152	75	314	233	78	0.645	0.784	133
6	135	122	90	90	62	69	335	314	94	598	581	97	0.226	0.210	93
7	138	140	101	75	55	73	371	245	66	640	438	68	0.216	0.320	148
8	144	130	90	75	72	96	377	305	81	639	555	87	0.225	0.234	104
9	158	120	76	106	61	58	414	223	54	726	391	54	0.218	0.307	141
10	129	136	105	71	53	75	392	300	77	754	625	83	0.171	0.218	127
Sub.Av.	141	130	92	83	61	74	378	277	74	671	518	78	0.211	0.258	123
11	147	117	80	78	46	59	406	207	51	812	431	53	0.181	0.271	150
12	143	129	90	128	50	39	457	410	90	802	788	98	0.178	0.164	92
13	130	114	88	55	44	80	515	299	58	805	482	60	0.161	0.237	147
14	145	80	55	76	31	41	487	169	35	854	313	37	0.170	0.256	151
15	134	122	91	50	33	66	422	298	71	861	648	75	0.156	0.188	121
Sub.Av.	140	112	81	77	41	57	457	277	61	827	532	65	0.169	0.223	132
16	120	107	89	111	84	76	550	228	41	932	407	44	0.129	0.263	204
17	167	138	83	83	45	54	534	458	86	1027	864	84	0.163	0.160	98
18	129	121	94	95	87	92	564	439	78	1064	784	74	0.121	0.154	127
19	140	129	92	113	80	71	770	440	57	1400	830	59	0.100	0.155	155
20	126	107	85	121	115	95	860	750	87	1483	1250	84	0.085	0.086	101
Sub.Av.	136	120	89	105	82	78	656	463	70	1181	827	69	0.120	0.164	137
Mean	141	126	90	75	51	70	427	292	70	748	528	73	0.286	0.357	131

MBP: Mean blood pressure.
GFR (In.): Glomerular filtration rate—(inulin clearance).
RPF: Clearance of para-aminohippurate.
RBF: Renal blood flow $= \frac{\text{RPF}}{\text{1-hematocrit}}$.
RVR: Renal vascular resistance = MBP/RBF.
C: Control value.
T: Tilt value.
%C: Tilt value expressed as per cent of control.

RESULTS

In order to determine the relationship of renal vascular impairment to renal hemodynamic alterations following tilting, these twenty patients have been arbitrarily divided (Table I) into four equal groups of five patients each in ascending order of control renal blood flow (RBF), since it was felt that the

effect on renal blood flow was more indicative of renal vasoconstriction than was the effect on glomerular filtration rate (GFR). In most instances, however, the changes in GFR closely paralleled the changes in renal blood flow. The values for the blood urea nitrogen content also fell in a progressive manner by this division. The effects of tilting on renal hemodynamics expressed in terms of per cent of control are furnished in the third column of Tables I and II. There was no significant difference in the response between any of the four groups of hypertensive patients. There was a 10 per cent drop in mean blood pressure in the tilted position but a greater drop in GFR, RPF, and renal blood flow, the latter indicating an increase in renal vascular resistance (Fig. 1). There was a parallel reduction in RPF and GFR due to tilting (Fig. 2).

TABLE II. EFFECT OF TILTING ON TMPAH, CREATININE CLEARANCE

PATIENT	BUN	CREATININE* CLEARANCE			TMPAH			GFR/TM			HCT		
		C	T	%C	C	T	%C	C	T	%C	C	T	%C
1	44	15	14	93	–	–	–	–	–	–	27	27	100
2	40	32	29	91	25	25	100	108	96	89	42	50	119
3	60	33	28	85	33	32	97	112	122	109	37	36	97
4	32	57	25	44	–	–	–	–	–	–	22	26	118
5	40	56	28	50	40	27	68	113	70	62	29	31	107
Sub.Av.		39	25	73	33	28	88	111	96	87	31	34	108
6	24	80	53	66	81	51	63	111	122	110	44	46	105
7	23	68	63	93	53	49	92	142	112	79	42	44	105
8	16	67	51	76	–	–	–	–	–	–	41	45	110
9	21	97	55	57	–	–	–	–	–	–	43	43	100
10	23	–	–	–	–	–	–	–	–	–	48	52	108
Sub.Av.		78	56	73	67	50	78	127	117	95	44	46	106
11	15	82	38	46	59	47	80	132	98	74	50	52	104
12	15	126	40	32	73	42	58	175	119	68	43	48	112
13	15	65	42	65	51	51	100	108	86	80	36	38	106
14	19	67	17	25	63	51	81	121	61	50	43	46	107
15	22	43	32	74	38	26	68	132	127	96	51	54	106
Sub.Av.		77	34	48	57	43	77	134	98	74	45	48	107
16	8	105	88	84	–	–	–	–	–	–	41	44	107
17	–	75	62	83	–	–	–	–	–	–	48	47	98
18	14	97	70	72	92	67	73	103	130	126	47	44	94
19	14	111	73	66	58	58	100	195	138	71	45	47	104
20	15	146	140	96	95	88	93	127	131	103	42	40	95
Sub.Av.		107	87	80	82	71	89	142	133	100	45	44	100
Mean		75	50	68	59	47	83	129	109	86	41	43	105

BUN: Blood urea nitrogen (milligram per cent).
*: Clearance of creatinine—milliliters per minute.
TmPAH: Maximum tubular excretory capacity for PAH (milligrams per minute).
GFR/Tm: Ratio of glomerular filtration rate (inulin clearance) to tubular excretory capacity for PAH.
HCT: Hematocrit (Wintrobe).
C: Control value.
T: Tilt value.
%C: Tilt value expressed as per cent of control.

Maximum tubular function (TmPAH) (Table II) was also significantly depressed indicating a reduction in the number of functioning nephrons. The decrease in GFR:TmPAH ratio, however, demonstrates that the decline in the number of nephrons was exceeded by the decrease in filtration rate since the

latter is more sensitive to changes in hemodynamics (Fig. 3). The hematocrit was not significantly altered although there was a trend toward a slight increase (5 per cent).

The rates of water and sodium excretion were depressed more than any other process studied (Table III). Potassium excretion was only slightly depressed. The reduction in water excretion during tilting was out of proportion to the fall

TABLE III. EFFECT OF TILTING ON RENAL EXCRETION OF WATER AND ELECTROLYTES

PATIENT	URINE VOLUME			SODIUM EXCRETION			POTASSIUM EXCRETION		
	C	T	%C	C	T	%C	C	T	%C
1	2.0	1.6	80	191	157	82	31	26	84
2	3.6	3.6	100	–	–	–	–	–	–
3	8.4	5.6	67	548	422	77	74	64	86
4	1.9	0.2	11	513	32	6	110	36	33
5	9.7	7.2	74	–	–	–	–	–	–
Sub.Av.	5.1	3.6	66	417	204	55	72	42	68
6	11.1	9.6	86	–	–	–	–	–	–
7	11.1	4.6	41	570	117	21	185	69	37
8	8.4	8.0	95	317	130	41	31	36	116
9	6.2	2.4	39	957	313	33	97	49	51
10	0.8	0.2	25	70	19	27	36	41	114
Sub.Av.	7.5	5.0	57	479	145	31	87	49	80
11	3.0	0.6	20	–	–	–	–	–	–
12	8.4	5.2	62	478	48	10	138	49	36
13	10.9	6.2	57	217	78	36	23	23	100
14	3.1	0.4	13	439	270	62	136	59	43
15	3.0	1.0	33	–	–	–	–	–	–
Sub.Av.	5.7	2.7	37	378	132	36	99	44	60
16	9.9	0.9	9	152	52	34	13	10	77
17	29.0	16.0	55	274	74	27	38	41	108
18	7.9	3.0	38	783	270	34	77	74	96
19	10.9	2.8	26	–	–	–	–	–	–
20	4.0	3.2	80	378	309	82	105	74	70
Sub.Av.	12.3	5.2	42	397	176	44	58	50	88
Mean	7.7	4.1	51	421	164	41	78	47	75

Urine Volume: expressed as milliliters per minute.
Sodium Excretion: expressed as microequivalents per minute.
Potassium Excretion: expressed as microequivalents per minute.
C: Control value.
T: Tilt value.
%C: Tilt value expressed as per cent of control.

TABLE IV. STATISTICAL SUMMARY OF EFFECTS OF TILTING ON RENAL HEMODYNAMICS*

FUNCTION	MEAN CHANGE ± S.E. OF MEAN	"t"	"p"
MBP	–14.9 ± 3.68	4.035	<0.01
GFR (in)	–23.6 ± 4.37	5.391	<0.01
GFR (cr)	–25.0 ± 4.82	5.176	<0.01
RPF	–134.6 ± 22.80	5.940	<0.01
RBF	–226.8 ± 40.40	5.607	<0.01
RVR	0.071 ± 0.022	3.151	<0.01
TmPAH	–11.31 ± 3.09	3.656	<0.01
U.V.	–4.41 ± 1.06	4.150	<0.01

*Statistical analysis by Dr. H. E. Rockwell. The changes in renal function due to tilting were analyzed by the application of "t" test to paired differences.

$$\text{"t"} = \bar{x}\sqrt{\frac{n\ (n-1)}{S_{x^2}}}$$

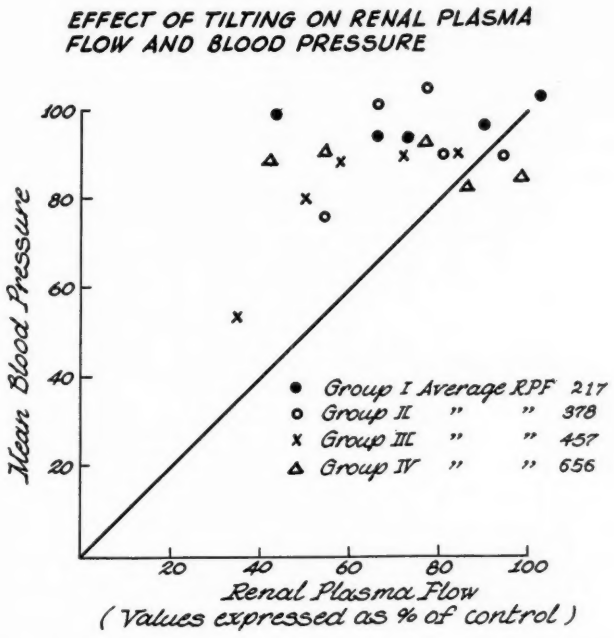

Fig. 1.—Effect of head-up tilting in patients with hypertension—mean blood pressure for each patient is plotted against renal plasma flow and the values after tilting are expressed in per cent of the control observations. There is a tendency toward blood pressure reduction, but renal plasma flow is reduced out of proportion, indicating an increase in renal vascular resistance.

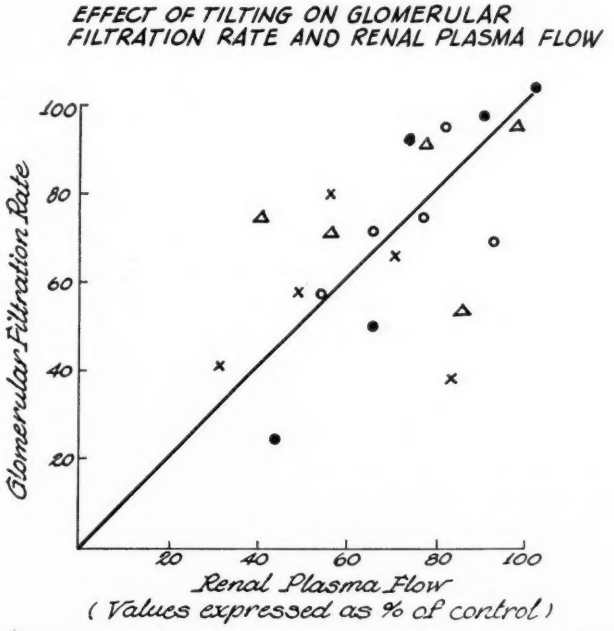

Fig. 2.—The effect of head-up tilt on glomerular filtration rate as compared to renal plasma flow in patients with hypertension. The values after tilt are expressed in per cent of the control observations. There is approximately an equivalent reduction in glomerular filtration rate and renal plasma flow. Symbols: ●, patients in Group 1; 0, patients in Group 2; x, patients in Group 3; △, patients in Group 4.

TABLE V. EFFECT OF AMBULATION ON RENAL HEMODYNAMICS

PATIENT	DIAGNOSIS	AGE	MEAN BLOOD PRESSURE (MM. Hg)		GLOMERULAR FILTRATION RATE* ML./MIN.		RENAL PLASMA FLOW ML./MIN.		TUBULAR EXCRETORY CAPACITY MG./MIN.		HEMATOCRIT		RENAL BLOOD FLOW ML./MIN.		RENAL VASCULAR RESISTANCE†	
			R	A	R	A	R	A	R	A	R	A	R	A	R	A
21	HVD	60	156	156	100	86	572	450	66	56	45	46	1040	833	0.150	0.187
22	MHVD	62	210	220	52	45	221	179	35	23	46	47	409	338	0.513	0.651
23	HCVD	56	160	160	90	86	445	387	72	71	45	47	809	730	0.198	0.219
24	HVD	58	123	133	67	73	570	509	62	57	42	43	983	893	0.125	0.149
25	HCVD	67	147	147	78	69	728	680	83	85	45	46	1324	1259	0.111	0.117
26	HCVD	60	160	150	52	33	255	177	48	47	42	46	440	328	0.364	0.457
27	HVD	42	160	160	74	63	355	280	77	62	41	42	602	483	0.266	0.331
Mean			159	161	73	65	449	380	63	57	44	45	801	695	0.247	0.302
Mean % of Supine Control‡				101		88		82		89		104		85		120

R: Recumbent.
A: Ambulatory.
HVD: Hypertensive vascular disease.
MHVD: Malignant hypertensive vascular disease.
HCVD: Hypertensive cardiovascular disease.

*Inulin clearance.

†Renal vascular resistance $= \frac{\text{Mean Blood Pressure}}{\text{Renal Blood Flow}}$.

‡Mean value for per cent of control of individual patients when control equals 100 per cent.

in GFR (Fig. 4). The effect on sodium excretion was similar to the effect on water excretion, the latter also declining out of proportion to the fall in GFR (Fig. 5). Although there was approximately a parallel decline in water and sodium excretion during tilting, the scatter was fairly wide (Fig. 6).

The results obtained from the ambulatory studies are recorded in Tables V and VI. In contrast to the response in the passively tilted position, when changing from the supine quiescent state to the upright ambulatory state, there was no alteration of mean blood pressure. However, GFR, RPF, and TmPAH were depressed, indicating renal vasoconstriction. The intensity of the constriction was not nearly as marked as was previously observed in the passively tilted position (Tables I and II). The response of electrolyte and water excretion to ambulation was also qualitatively similar to the response to passive tilt, but again was not so marked.

TABLE VI. EFFECT OF AMBULATION ON WATER AND ELECTROLYTE EXCRETION

	URINE VOLUME (ML./MIN.)		SODIUM EXCRETION (μEQ/MIN.)		POTASSIUM EXCRETION (μEQ/MIN.)		PLASMA CONCENTRATION (MEQ./L.)			
							SODIUM		POTASSIUM	
PATIENT	R	A	R	A	R	A	R	A	R	A
21	9.4	7.4	287	191	121	138	139	134	3.3	3.2
22	17.0	16.0	361	204	236	200	129	124	3.2	3.0
23	27.0	11.0	565	204	146	282	140	133	3.7	3.7
24	16.0	17.0	191	217	162	164	140	140	3.0	3.0
25	4.5	5.6	226	200	231	269	140	132	4.1	4.2
26	9.0	2.7	1043	1043	205	115	136	125	3.7	3.6
27	10.0	7.6	870	522	128	103	140	132	2.9	3.1
Mean	13.3	9.6	506	369	176	182	138	131	3.4	3.4
*Mean % of Supine Control		79		74		107		95		100

R: Recumbent.
A: Ambulatory.
*Mean for per cent of control of individual observations.

DISCUSSION

When a person assumes a quiet upright or a tilted position, progressive venous pooling of blood leads to decreased cardiac output and reflex vasoconstriction resulting from the stimulation of homeostatic receptors throughout the body. Vasomotor changes (vasoconstriction) in the kidney seem to be responsible for most of the observed changes in renal function. This renal vasoconstriction appears to result from vascular stimuli designed to maintain arterial blood pressure[9] and is probably mediated through the sympathetic nervous system, although vasoconstricting humoral agents may be partly responsible. Similar changes induced by mechanical aortic occlusion in dogs have been observed.[11] Our results in tilting patients with hypertension are in close agreement with those found in normal individuals as reported in the literature,[1-5] which indicate an increase in renal vascular resistance in the tilted position. Although there was a slight reduction in blood pressure there was a much greater depression in renal blood flow, indicating an increase in renal vascular resistance (Fig. 1). The absence of change in filtration fraction indicates that this is due to vasoconstriction, which is approximately equal in the afferent and efferent renal

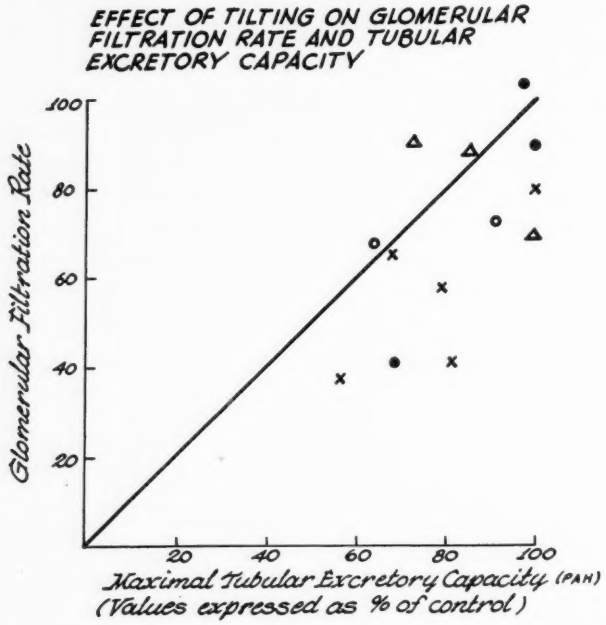

Fig. 3.—Effect of tilting on glomerular filtration rate as compared to maximal tubular function (TmPAH) in patients with hypertension. Values after tilt are expressed in per cent of control observations. Generally, glomerular filtration rate is reduced out of proportion to TmPAH. Symbols: ●, patients in Group 1; 0, patients in Group 2; x, patients in Group 3; △, patients in Group 4.

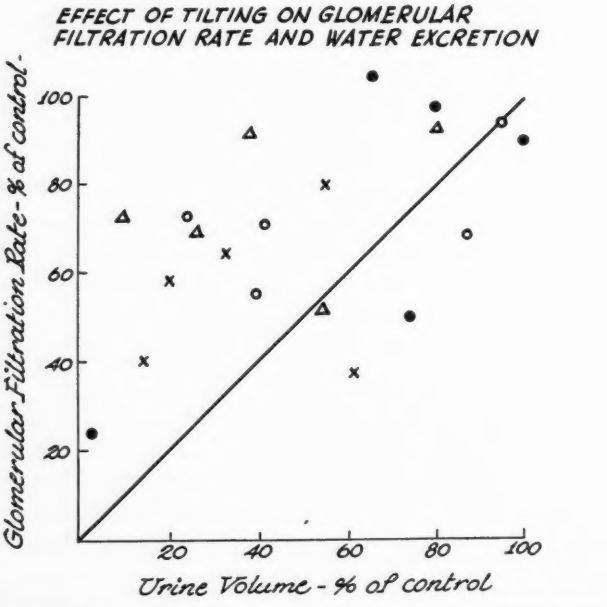

Fig. 4.—Effect of tilting on urine volume as compared to glomerular filtration rate in patients with hypertension. Values are expressed in per cent of control observations. There was a reduction in urine volume out of proportion to the depression in glomerular filtration rate. Symbols: ●, patients in Group 1; 0, patients in Group 2; x, patients in Group 3; △, patients in Group 4.

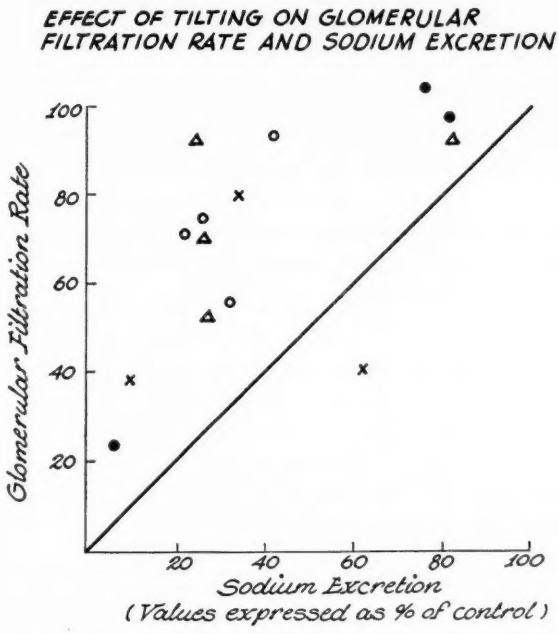

Fig. 5.—Effect of tilting on sodium excretion as compared to glomerular filtration rate in patients with hypertension. Values after tilt are expressed as per cent of control observations. Sodium excretion is depressed out of proportion to the reduction in glomerular filtration rate. Symbols: ●, patients in Group 1; 0, patients in Group 2; x, patients in Group 3; △, patients in Group 4.

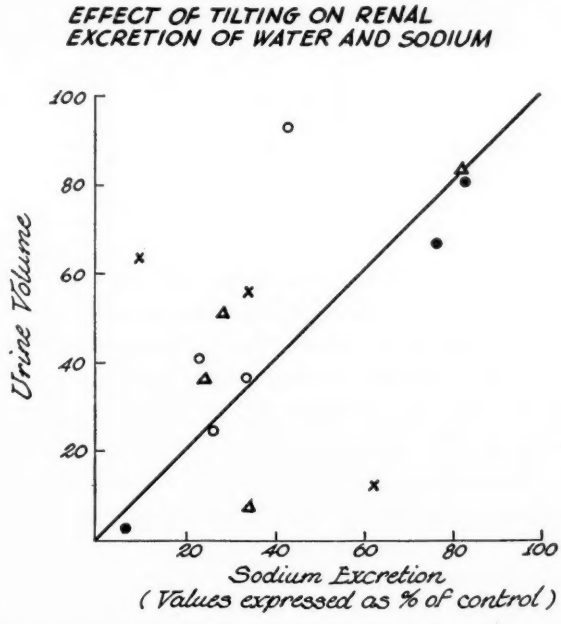

Fig. 6.—Effect of tilting on urine volume as compared to sodium excretion in patients with hypertension. Values after tilting are expressed in per cent of control observations. There is usually an equivalent reduction in urine volume and in sodium excretion. Symbols: ●, patients in Group 1; 0, patients in Group 2; x, patients in Group 3; △, patients in Group 4.

arterioles (Fig. 2). The decrease in TmPAH also indicates that the number of functioning nephrons has been reduced by the vasoconstriction which follows the head-up tilt. Since there is a decrease in the GFR:Tm ratio, there is not only a decrease in number of functioning nephron units but a decrease in filtration in those remaining units. These effects are also observed during ambulation but the vasoconstrictor response is much less marked (Table VIII).

TABLE VII. STATISTICAL SUMMARY OF EFFECTS OF AMBULATION ON RENAL HEMODYNAMICS*

FUNCTION	MEAN CHANGE ± S.E. OF MEAN	"t"	"p"
MBP	1.4 ± 2.61	0.549	n.s.
GFR (in)	−8.3 ± 3.00	2.763	<0.05
RPF	−69.1 ± 10.10	6.846	<0.01
RBF	−106.1 ± 18.45	5.753	<0.01
RVR	0.055 ± 0.018	3.085	<0.05
TmPAH	−6.0 ± 2.43	2.470	<0.05
U.V.	−3.7 ± 2.27	1.619	n.s.

*Statistical analysis by J. B. Hummel, M.A. The changes in renal function due to ambulation were analyzed by the application of "t" test to paired differences.
S.E. = Standard error.

TABLE VIII. SUMMARY OF RENAL RESPONSE TO AMBULATION AS COMPARED TO 60-DEGREE HEAD-UP PASSIVE TILT EXPRESSED AS MEAN VALUES

FUNCTION STUDIED	RESPONSE TO AMBULATION			RESPONSE TO TILT		
	R	A	A/R × 100	R	T	T/R × 100
Mean blood pressure (mm. Hg)	159	161	101	141	126	89
Glomerular filtration rate (ml./min.)	73	65	89	75	51	68
Renal plasma flow (ml./min.)	449	380	85	427	292	68
Tubular excretory capacity (mg./min.)	63	57	90	59	47	80
Hematocrit	44	45	102	41	43	105
Renal blood flow (ml./min.)	801	695	87	748	528	71
Renal vascular resistance	0.247	0.302	122	0.286	0.357	125
Urine volume (ml./min.)	13.3	9.6	72	7.7	4.1	53
Sodium excretion (μEq/min.)	506	369	73	421	164	39
Potassium excretion (μEq/min.)	176	182	103	78	47	60

R: Recumbent.
A: Ambulatory.
T: Head-up tilt.

The depression of water excretion out of proportion to the depression of glomerular filtration rate, renal plasma flow, or tubular excretory capacity suggests that there may be factors other than vasoconstriction in operation.[6-8] The reduction in sodium excretion in the current study seems to be closely related to the depression in urine volume. However, others[12] have shown that the antidiuretic hormone has no specific effect on the reabsorption of sodium. Mudge and associates[13] have emphasized that the sodium concentration in the renal tubular urine is probably an important factor in the rate at which this ion is transported. Interest has recently returned to the neurogenic factors concerned in water and sodium excretion, and evidence has been presented in favor of some neurogenic effects[14, 15] on tubular reabsorption of sodium and water. Anesthetized dogs, when acutely denervated, show an increased water and salt excretion without change in GFR or RPF. Unanesthetized dogs,

when acutely denervated, show a decreased water excretion without change in GFR or RPF.[14] Hydergine,* an adrenergic blocking agent given to anesthetized animals, increases water and salt excretion with little effect on potassium excretion.[15] Another group[16] has shown that norepinephrine decreased RPF without changing GFR, increased the tubular reabsorption of sodium and potassium, and increased water excretion. This effect could not be abolished by high spinal anesthesia or the hypoadrenocortical state.

It is readily seen that the extra renal factors controlling sodium and water excretion are multiple and have been identified only in part. The renal factors are still subject to investigation but are even more difficult to dissect due to the limitations of available techniques.[17]

There was no significant difference in the reactivity of the severely damaged kidney as compared to the normal ones since all groups of patients showed similar responses in hemodynamics as well as in water and electrolyte excretion. Furthermore, the changes in this group of patients with hypertensive vascular disease closely resemble those seen in normotensive patients. This suggests that the nephrons that remain functional react in a relatively normal manner.

It can be seen that there was a considerably greater depression of renal functions in the passively tilted position than in the ambulatory state. Renal blood flow for the group was reduced to 71 per cent in the passively tilted position but only to 87 per cent in the upright ambulatory state. This response is also reflected in a slightly greater increase in renal vascular resistance in the passively tilted position as compared to the ambulatory state. These observations are of at least academic importance since ambulation is a physiologic state as applied to the activities of everyday life whereas the passively tilted position is not.

SUMMARY

Twenty-seven hypertensive patients have been studied in order to evaluate the effects of a 60-degree head-up tilt (twenty patients) and ambulation (seven patients) on renal hemodynamics and the renal excretion of water and electrolytes.

There is an increase in renal vascular resistance with a decrease in filtration in a decreased number of nephrons. There is also a decrease in urine formation and sodium excretion greater than the increase in renal vascular resistance. The significance of this is discussed.

The ambulatory state is qualitatively similar to head-up tilting in its ability to increase renal vascular resistance and to depress renal function but it is quantitatively less severe.

The renal vessels in patients with hypertension respond in essentially the same manner and to the same degree as do those of normal patients with normal kidneys. Renal damage does not alter the ability of the vessels of the nephrons that remain functional to constrict under stress of ambulation or the passively head-up tilted position.

*An equal mixture of dihydroergocornine, dihydroergokryptine, and dihydroergocristine.

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CORRECTION FOR COLOR DIFFERENCES BETWEEN STANDARDS AND URINE EXTRACTS AND THEIR KETONIC FRACTIONS IN THE CALLOW-ZIMMERMANN REACTION

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INTRODUCTION

IN STUDYING urinary 17-ketosteroid excretion of persons with a variety of diseases, we were impressed by the diversity of the colors generated from the various specimens. Because variations in the spectral purity of colors employed for analysis make the results ambiguous and the comparison of data obtained on different specimens and individuals hazardous, we felt further study of this subject to be indicated. The results are presented here.

METHODS

We used only 24-hour urine specimens. These were hydrolyzed the day collection was completed as follows: to the urine which had just been heated to boiling we added as rapidly as possible 10 per cent of its volume of concentrated hydrochloric acid. The acidified urine was then reheated to the boiling point, and the ten-minute hydrolysis period counted from the time the specimen began to boil the second time. After completion of hydrolysis the specimen was cooled immediately to room temperature under running tap water and extracted four times with one-fifth of its volume of freshly distilled U.S.P. ether from which peroxides had been removed before distillation by washing with acidified aqueous ferrous sulfate. The combined ether extracts were washed with 0.5N aqueous sodium hydroxide so that two successive washings had a pH of 12 or more. The washings were tested with Accutint wide-range B indicator paper. The alkaline ether solution we washed with distilled water until the pH of the washings was 7 to 8. Ether was distilled at a water bath temperature of less than 60° C. under water-pump vacuum. Residual water was codistilled with distilled methanol in vacuo, and the residue was finally dried at room temperature in a vacuum desiccator to remove the last trace of moisture. During the interval between obtaining and analyzing an extract, it was always stored as a dry residue in a deep freezer. During periods of a few days only minimal changes occur under these conditions. Extract residues were dissolved in appropriate volumes of absolute ethanol for analysis. All ethanol used in carrying out the Zimmermann reaction was prepared by overnight refluxing with potassium hydroxide (50 Gm. per liter) followed by distillation. As soon as samples had been analyzed for 17-ketosteroids, aliquots for the Girard separation were withdrawn from the original ethanol solution and dried in vacuo. The Girard separation was done on these portions on the following day. The ketonic and nonketonic fractions thus obtained were analyzed one day later.

We did all Zimmermann reactions by the Callow[1] method with volumes adjusted to 0.5 ml. rather than the usual 0.2 ml. In the first half of the experimental work Eastman Kodak Company's White Label *m*-dinitrobenzene (*m*-DNB), m.p. 89.5° to 91.0° C., remelt 90° to 91° C. (uncorrected, Fisher-Johns melting point block) was purified by the procedure described by Callow,[1] giving a slightly purer material, m.p. 90.0° to 91.5° C.

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Because this purified material was not superior to the unpurified *m*-DNB, we used the latter during the final part of the experimental work. *m*-DNB was made up to a 2 per cent solution in absolute ethanol. Potassium hydroxide (2.50 ± 0.03N) in absolute ethanol was freshly prepared just before use because storage significantly elevated the reagent blank. We set up the following tubes each time: (1) Reagent blank: 0.5 ml. ethanol, 0.5 ml. KOH, 0.5 ml. *m*-DNB. (2) Standard*: 0.1 mg. standard in 0.5 ml. ethanol, 0.5 ml. KOH, 0.5 ml. *m*-DNB. (3) Standard: 0.3 mg. standard in 0.5 ml. ethanol, 0.5 ml. KOH, 0.5 ml. *m*-DNB. (4) Urine extract: 0.5 ml. extract, 0.5 ml. KOH, 0.5 ml. *m*-DNB. (5) Urine extract blank: 0.5 ml. extract, 0.5 ml. KOH, 0.5 ml. ethanol. Tube 4 was run in duplicate for each specimen. We analyzed four urine extracts in this way during each run. Finally, Tubes 3, 2, and 1 were repeated in that order. In setting up a run we added only the first two ingredients to all tubes initially. (The urine extract blanks, Tube 5, were set up in toto.) We then brought the tubes and contents to 20° C. in a bath. Then *m*-DNB was added to the first pair of tubes (1 and 2) and a 45-minute interval was timed from this addition. At ten-minute intervals we added *m*-DNB to successive pairs of tubes, until it had been added to all tubes except urine extract blanks. After 45 minutes we diluted each of the first pair of tubes with 20 ml. of 70 per cent ethanol and measured their absorbancies. Successive pairs of tubes were then diluted and measured at ten-minute intervals so that each pair had a 45-minute incubation period at 20° C. after addition of the *m*-DNB.† We measured absorbancies with the Beckman Model DU Spectrophotometer‡ at 425, 510, and 595 mμ and with the Fisher Electrophotometer at 425 and 525 mμ using appropriate filters.

Girard procedures were done according to Pincus and Pearlman,[2] except that 150 mg. of Girard's reagent T were used instead of 100. We did all evaporations in vacuo, and appropriate dilutions of the ketonic and nonketonic fractions were analyzed by the Zimmermann reaction as described for crude 17-ketosteroid extracts previously.

The results were computed as follows:

1. Uncorrected 17-ketosteroid content of crude urine extracts:
   a. (A_s)§ $_{\text{standard 510}} - (A_s)_{\text{reagent blank 510}} =$ value for plotting standard curve at 510 mμ.
   b. $(A_s)_{\text{17-ketosteroid 510}} - [(A_s)_{\text{reagent blank 510}} + (A_s)_{\text{urine extract blank 510}}] =$ value to be read off standard curve to give amount of 17-ketosteroid taken for analysis.
2. 17-Ketosteroid content corrected by comparing the absorption peak of the unknown with that of the standard:
   a. $(A_s)_{\text{reagent blank 510}} - \frac{1}{2}[(A_s)_{\text{reagent blank 425}} + (A_s)_{\text{reagent blank 595}}] =$ value to be subtracted from result 2b.
   b. $(A_s)_{\text{standard 510}} - \frac{1}{2}[(A_s)_{\text{standard 425}} + (A_s)_{\text{standard 595}}] =$ value for plotting absorption peak standard curve, after 2a has been subtracted.
   c. $(A_s)_{\text{urine extract blank 510}} - \frac{1}{2}[(A_s)_{\text{urine extract blank 425}} + (A_s)_{\text{urine extract blank 595}}] =$ value to be subtracted from 2d.

*A sample of dehydroisoandrosterone acetate, m.p. 168° to 169° to 170° C. (uncorrected, Fisher-Johns melting point block) was used as the reference standard throughout this investigation. We wish to express thanks to the Schering Corporation for generously supplying this material.

†The KOH was added to urine extract blanks at the same time it was added to the corresponding urine extract. Likewise, as described previously, the blank was diluted with 70 per cent ethanol at the same time as, or within 10 minutes of, the dilution of the corresponding urine extract. This appears to be necessary in order to get optimal correlation between the pigments in the two tubes. Incubation with KOH causes a fall in the intrinsic color of the urine extract. The greatest fall in absorbancy occurs in the region of 510 mμ, with a much smaller decrease at 425 mμ and least at 595 mμ. Since the maximum absorbancy change is in the most critical region, urine extracts and their corresponding blanks should be treated as uniformly as possible in regard to incubation with KOH. The most rapid fall in absorbancy of these pigments occurs when KOH is first added, and has fairly well leveled off after 45 minutes.

‡All data in this paper were obtained with the Beckman Spectrophotometer unless the Fisher instrument is specified.

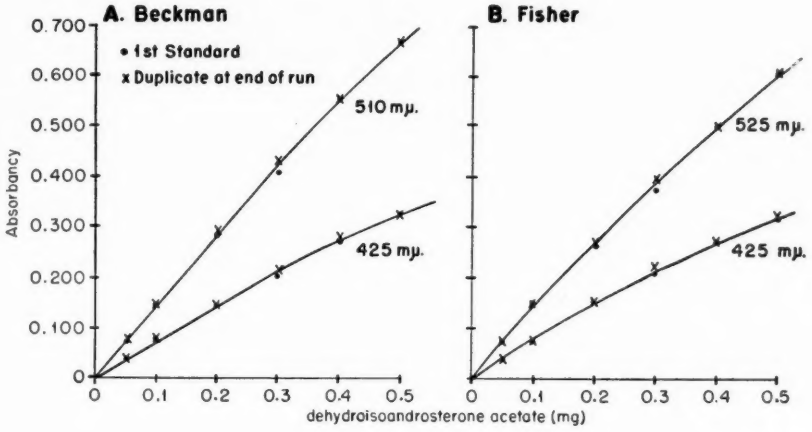
§The symbols are defined in Stearns[3] (pp. 307 and 357). We have used those recommended by the National Bureau of Standards Letter Circular LC-857 unless otherwise specified. The total measured absorbancies are written as A_s.

d. (A_s) 17-ketosteroid 510 $-$ ½ [(A_s) 17-ketosteroid 425 + (A_s) 17-ketosteroid 595] = total absorption peak of 17-ketosteroid.

e. 2d $-$ [2a + 2c] = absorption peak of 17-ketosteroidal chromogens; when read off corrected standard curve gives amount of 17-ketosteroid taken for analysis.

3. The uncorrected 17-ketosteroid content of Girarded ketonic fractions was computed as in Number 1 using a ketonic fraction blank in place of the urine extract blank.

4. The corrected 17-ketosteroid content of Girarded ketonic fractions was computed as in Number 2 using the ketonic fraction blank in place of the urine extract blank.



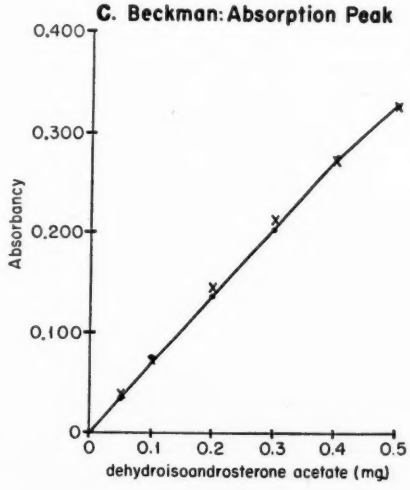


Fig. 1.—Standard curves.

Appropriate factors for converting the amount of 17-ketosteroid taken for analysis to total 24-hour excretion were used in each instance previously.

Standard curves for the Beckman at 425 and 510 mμ and the Fisher at 425 and 525 mμ are plotted in Fig. 1, *A* and *B*. The Beckman absorption peak standard curve is plotted in Fig. 1, *C*.

In the urine hydrolysis, acid was added to boiling urine to minimize variations in the lengths of time different specimens were heated with acid. In some cases in order to get enough 17-ketosteroid it was necessary to hydrolyze 2 L. of urine. By this procedure a 500 ml. specimen, which can be quickly brought to a boil, is in contact with hot acid for nearly the same time as a 2 L. specimen.

RESULTS AND DISCUSSION

In the colorimetric analysis of 17-ketosteroids the ultimate requirement is to compare the color generated in an unknown extract with the color of the standard. But only "comparable" colors should be compared. Unfortunately, only very rarely is the spectral purity of the color generated in an unknown identical with that of the standard. That the two colors are almost never identical in the case of 17-ketosteroids is obvious when one considers that a single compound is used as the standard and that any unknown consists of a complex mixture of known and undiscovered 17-ketosteroids. Each of the various 17-ketosteroids has its own individual absorption spectrum (color)

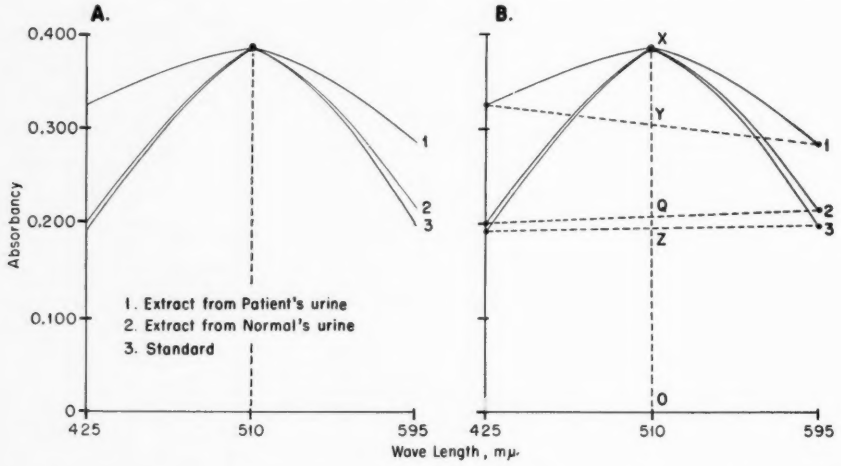


Fig. 2.—Zimmermann reaction absorption curves. Reagent and urine extract blanks have been subtracted.

when subjected to the Zimmermann reaction.[4, 5] There are also non-17-ketosteroidal chromogenic substances in urine extracts which generate still other colors in the Zimmermann reaction and are not correctable by blanks. Since direct comparison of the colors of extracts with standards is wrong, how can one obtain reasonably precise and accurate results which can validly be compared with each other? We approached the problem as follows: Fig. 2, *A*, Curves 1 and 2 are representative of the types of colors different urine extracts develop in the Zimmermann reaction. The absorption spectrum of the standard is Curve 3 in Fig. 2, *A*. When color intensity is measured at 510 mμ only, all three specimens contain the same amount of 17-ketosteroid. This is obviously wrong. Curves 1 and 3 are more logically compared as in Fig. 2, *B*, where the indicated readings are made at the three wave lengths, so that the absorptions XY and XZ may be computed. The 17-ketosteroid contents of the standard

and the unknown are best related as XZ is to XY. If the same procedure is carried out for Curve 2, the 17-ketosteroid contents represented by Curves 1 and 2 are related as XY is to XQ. When the determinations shown by Curves 1 and 2 are made on different occasions the data obtained are comparable because both are computed in terms of the standard, XZ. We found this the best way to measure the 17-ketosteroidal chromogen content of urine extracts and to detect a change in pattern from Curve 1 to Curve 2 in an individual excreting 17-ketosteroidal chromogens.

How can this approach be evaluated from the standpoint of validity and accuracy and put in proper perspective regarding the magnitude of the errors when simple measurement is made at one wave length as in Fig. 2, *A*? First, the "true" value for the 17-ketosteroid content of an extract must be established for use as a reference point. Then correlation with this "true" value is the criterion for validity when results computed as in Fig. 2, *A* are compared with data computed as in Fig. 2, *B*. Unfortunately this "true" value cannot be determined. As an approximation we may assume that if a 17-ketosteroid extract is separated into ketonic and nonketonic fractions by the Girard procedure, the 17-ketosteroid content of the ketonic fraction is the "true" 17-ketosteroid content of the crude extract. This assumption has been used[6-8] in other investigations. A study of such ketonic fractions from a variety of people, however, reveals absorption curves of Types 1, 2, and 3, Fig. 2, *A*. Occasionally the ketonic fraction will have a higher peak color than the standard. Thus, the objections to uncorrected analyses (at one wave length) of crude extracts also apply to the ketonic fractions obtained from them. Other problems inherent in the use of the Girard procedure are discussed later.

This brings us back to our starting point. To get 17-ketosteroid analyses which may confidently be compared with one another, even data on the ketonic fraction, which was to be the "true" value, must be computed using measurements at three wave lengths. However, when the corrected and uncorrected data obtained in this way are tabulated, useful relations are observed. Table I gives data on normals. Uncorrected values on crude extracts (Column 2) are always greater than data obtained by correcting them (Column 4), or by the Girard separation, whether analyses on the ketonic fraction are uncorrected or corrected (Column 3). These differences are not significant as far as altering the clinical interpretation of 17-ketosteroid analyses. There is greater purity of Zimmermann color after Girard separation, because corrected values on the ketonic fractions are usually close to the uncorrected ones (Column 3). There is only fair agreement between data obtained on the ketonic fractions and data obtained by correcting the values from the crude extract (Column 4).

In Table II data on patients with diseases as indicated (Column 2) are presented. There is frequently a significant difference between the uncorrected value on the crude extract (Column 3) and data obtained by correction (Column 5) and/or Girard separation (Column 4). Even in those instances where the uncorrected values on the crude extracts do agree with the other

data, simple inspection of Zimmermann colors made one uncertain as to whether 17-ketosteroids were present. The 17-ketosteroid colors were completely obscured by the high blanks and chromogens, so that only by making the measurements at three wave lengths could one be certain of the existence of a peak. Greater purity of Zimmermann color results after Girard separa-

TABLE I. 17-KETOSTEROID ANALYSES ON NORMAL PERSONS (MG./24 HR.)

			2. CRUDE URINE EXTRACT	3. GIRARDED KETONIC FRACTION		4. CRUDE URINE EXTRACT
					CORRECTED BY ABSORPTION PEAK	
1. PERSON	SEX	AGE	UNCORRECTED			
H. 68	♂	33	13.5	12.7	11.6	12.4
H. 68A	♂	33	15.6	12.1	11.9	13.5
H. 70	♂	33	19.0	16.2	15.1	17.5
R. 69	♂	31	21.5	19.8	19.8	20.3
R. 69A	♂	31	24.0	20.5	20.0	21.4
R. 71	♂	31	31.1	27.7	25.5	27.5
B. 106	♂	27	29.7	27.3	25.9	27.1
B. 117	♂	27	28.1	21.9	21.2	25.5
L. 112	♀	25	19.1	18.6	17.9	17.4
R. 113	♀	27	9.6	8.1	7.7	8.2
J. II-28	♀	28	8.7	7.0	6.9	8.1
F. II-32	♀	26	9.4	7.4	6.8	7.4
K. 110	♂	9	1.4	1.2	1.1	0.7
P. 109	♂	6	1.1	0.9	0.8	0.6

TABLE II. 17-KETOSTEROID ANALYSES ON PATIENTS (MG./24 HR.)

				3. CRUDE URINE EXTRACT	4. GIRARDED KETONIC FRACTION		5. CRUDE URINE EXTRACT
						CORRECTED BY ABSORPTION PEAKS	
1. PATIENT	SEX	AGE	2. DISEASE	UNCORRECTED			
O. W. 80-1	♂	48	Laennec's cirrhosis, diabetes	7.6	5.0	3.8	4.6
O. W. 80-2	♂	48	Laennec's cirrhosis, diabetes	6.9	5.1	3.8	4.2
R. K. II-20	♂	39	Laennec's cirrhosis	3.8	1.8	1.2	1.0
R. K. II-24	♂	39	Laennec's cirrhosis	3.4	2.9	1.2	0.8
J. C. II-22	♂	58	Chronic adrenal insufficiency	3.5	2.9	2.4	2.2
F. K. 83	♂	53	Carcinoma of head of pancreas	9.3	6.1	4.9	3.7
L. O. 84	♂	42	Acute infectious hepatitis	2.9	3.0	2.3	1.7
G. G. 88	♂	19	Amyloidosis	1.1	0.7	0.7	0.6
G. G. 101	♂	19	Amyloidosis	1.0	1.0	0.8	0.6
G. G. 105	♂	19	Amyloidosis	2.3	2.0	1.5	1.5
R. P. 116	♂	23	Post hepatitis	21.6	18.0	17.0	19.9
B. H. 87	♀	50	Laennec's cirrhosis, cardiac failure	2.9	1.9	0.9	1.3
B. H. 99	♀	50	Laennec's cirrhosis, cardiac failure	6.1	6.2	6.9	6.2
A. S. 96	♀	28	Laennec's cirrhosis	3.7	3.0	3.0	3.1
A. S. 100	♀	28	Laennec's cirrhosis	4.0	2.8	2.4	2.9
N. C. II-34	♀	70	Biliary cirrhosis	2.0	1.2	0.8	1.2
L. L. I-138	♀	48	Laennec's cirrhosis	2.1	1.1	1.0	0.8
D. N. 107	♀	17	Amenorrhea, hirsutism	15.3	14.3	13.7	13.6
J. T. 111	♀	29	Diabetes, thyrotoxicosis	6.6	5.0	4.3	5.0
A. S. 119	♀	53	Cardiac failure, cardiac cirrhosis	1.4	1.1	0.9	1.0

tion, and the agreement between corrected data on the crude extract (Column 5) and results on ketonic fractions (Column 4) is certainly not remarkable.

Effects of the Girard Procedure on Crude Urine Extracts.—Previous observation of its failure to separate specifically ketonic and nonketonic substances[9] suggested investigation of the Girard procedure. We hoped to find the reason why the corrected crude extract analysis does not agree with its ketonic fraction analysis, either uncorrected or corrected (Tables I and II).

All crude urine extracts are colored. Color generated by the Zimmermann reaction on the extract is superimposed on these intrinsic colors. All urine extracts behave as follows in the Girard procedure: Color always remains in the aqueous phase after removal of both nonketonic and ketonic fractions. Absorbancy measurements on this aqueous phase cannot be interpreted, but the following question may be asked: Does pigment remaining in the aqueous phase represent destruction of intrinsic color, of non-17-ketosteroidal chromogens, of 17-ketosteroidal chromogens, or of them all?

TABLE III. URINE EXTRACT BLANKS COMPARED WITH THE SUM OF THE KETONIC AND NONKETONIC FRACTION BLANKS FROM THE SAME CRUDE EXTRACTS*

1. SPECIMEN	2. KETONIC BLANK	3. NONKETONIC BLANK	4. 2 + 3	5. URINE EXTRACT BLANK
H. 68	0.006	0.017	0.023	0.061
H. 68A	0.008	0.015	0.023	0.038
H. 70	0.003	0.008	0.011	0.017
R. 69	0.019	0.035	0.054	0.065
R. 69 (repeat)	0.005	0.013	0.018	0.065
R. 69A	0.008	0.012	0.020	0.049
R. 71	0.000	0.000	0.000	0.032
R. 71 (repeat)	0.000	0.003	0.003	0.032
B. 106	0.004	0.005	0.009	0.024
B. 106 (repeat)	0.005	0.005	0.010	0.024
B. 117	0.008	0.010	0.018	0.035
L. 112	0.021	0.014	0.035	0.084
R. 113	0.007	0.016	0.023	0.057
K. 110	0.030	0.063	0.093	0.222
P. 109	0.034	0.042	0.076	0.156
O. W. 80-1	0.035	0.075	0.110	0.147
O. W. 80-2	0.022	0.044	0.066	0.086
F. K. 83	0.008	0.034	0.042	0.075
L. O. 84	0.003	0.015	0.018	0.099
G. G. 101	0.020	0.024	0.044	0.135
G. G. 105	0.029	0.024	0.053	0.107
R. P. 116	0.021	0.056	0.077	0.126
B. H. 87	0.000	0.000	0.000	0.014
B. H. 99	0.022	0.043	0.065	0.098
A. S. 96	0.015	0.031	0.046	0.055
A. S. 100	0.003	0.011	0.014	0.027
D. N. 107	0.051	0.046	0.097	0.153
J. T. 111	0.011	0.007	0.018	0.071
A. S. 119	0.022	0.024	0.046	0.095
F. Z. 79	0.041	0.126	0.167	0.240

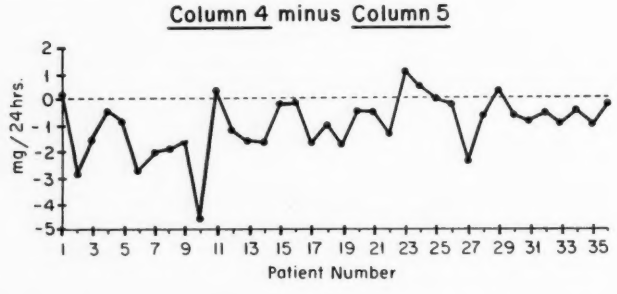
*All fractions of a given sample are at the same concentration and all measurements are at 510 mμ.

Destruction of intrinsic color is demonstrated in Table III. The urine extract blank measured when the crude 17-ketosteroid extract is analyzed is shown in Column 5. This extract is then split into ketonic and nonketonic fractions and both of their blanks (Columns 2 and 3, respectively) are meas-

TABLE IV. UNCORRECTED 17-KETOSTEROIDS FROM CRUDE EXTRACTS COMPARED WITH THE SUM OF THE UNCORRECTED KETONIC AND NONKETONIC CHROMOGENS FROM THE SAME EXTRACT (MG./24 HR.; ALL MEASUREMENTS AT 510 Mμ; ALL BLANKS SUBTRACTED)

1. SPECIMEN	2. KETONIC FRACTION	3. NONKETONIC FRACTION*	4. 2 + 3	5. CRUDE URINE EXTRACT
1. H. 68	12.7	1.0	13.7	13.5
2. H. 68A	12.1	0.6	12.7	15.6
3. H. 70	16.2	1.2	17.4	19.0
4. R. 69	19.8	1.3	21.1	21.5
5. R. 69 (repeat)	19.8	0.9	20.7	21.5
6. R. 69A	20.5	0.6	21.1	24.0
7. R. 71	27.7	1.4	29.1	31.1
8. R. 71 (repeat)	27.7	1.5	29.2	31.1
9. B. 106	27.3	0.8	28.1	29.7
10. B. 117	21.9	1.4	23.3	28.1
11. L. 112	18.6	0.7	19.3	19.1
12. R. 113	8.1	0.4	8.5	9.6
13. J. II-28	7.0	0.2	7.2	8.7
14. F. II-32	7.4	0.4	7.8	9.4
15. K. 110	1.2	0.1	1.3	1.4
16. P. 109	0.9	0.1	1.0	1.1
17. O. W. 80-1	5.0	0.9	5.9	7.6
18. O. W. 80-2	5.1	0.8	5.9	6.9
19. R. K. II-20	1.8	0.2	2.0	3.8
20. R. K. II-24	2.9	0.1	3.0	3.4
21. J. C. II-22	2.9	0.1	3.0	3.5
22. F. K. 83	6.1	1.9	8.0	9.3
23. L. O. 84	3.0	1.0	4.0	2.9
24. G. G. 88	0.7	0.9	1.6	1.1
25. G. G. 101	1.0	0.0	1.0	1.0
26. G. G. 105	2.0	0.2	2.2	2.3
27. R. P. 116	18.0	1.1	19.1	21.6
28. B. H. 87	1.9	0.4	2.3	2.9
29. B. H. 99	6.2	0.3	6.5	6.1
30. A. S. 96	3.0	0.1	3.1	3.7
31. A. S. 100	2.8	0.4	3.2	4.0
32. N. C. II-34	1.2	0.3	1.5	2.0
33. L. L. I-138	1.1	0.1	1.2	2.1
34. D. N. 107	14.3	0.6	14.9	15.3
35. J. T. 111	5.0	0.7	5.7	6.6
36. A. S. 119	1.1	0.2	1.3	1.4

*Corresponding specimens in Columns 3 and 5 are at the same concentration.



ured at the same concentration as was present in the original extract. The sum of the ketonic and nonketonic fraction blanks (Column 4) is always less than the urine extract blank. The Girard procedure partially destroys the color of extracts. For this reason appropriate blanks must be measured on crude extracts and ketonic fractions if the analyses are to be compared. Since this was done as in "Methods," the source of the discrepancy between corrected crude extract and ketonic data (Tables I and II) is not here.

Destruction of chromogens in the Girard procedure is shown in Table IV. If only the various blanks were bleached, the sum of chromogenic material in the ketonic and nonketonic fractions should equal the crude 17-ketosteroid analysis (all measurements made at 510 mμ only, with appropriate reagent, urine extract, ketonic and nonketonic blanks subtracted). Destruction of chromogenic material does occur because the sum (Column 4) is significantly lower than the original total (Column 5). Improved chromogenicity might be expected in the ketonic fraction after Girard separation since this procedure affords considerable purification. Thus, if creatinine were a major contaminant as suggested by Zimmermann,[10] one would find more chromogenicity after the separation, because absorbancies of mixtures of androsterone and creatinine were found "not to be the sum of the absorbancies alone, but 24% less at 530 mμ." It may be that enough destruction occurs to offset any gain in chromogenicity from purification. However, the addition of known amounts of pure 17-ketosteroids to crude urine extracts containing known amounts of 17-ketosteroids has given quantitative recoveries of added steroid.[7, 11] Also pure dehydroisoandrosterone added to the chromogens shows essentially the Zimmermann color development of the pure compound.[6] If this is true (cf. Reference 12), interference with Zimmermann color development of 17-ketosteroids by the usual urine extract contaminants is unlikely.

Observations summarized in Table IV could well contribute to the discrepancy between corrected crude extract data and results on the ketonic fractions (Tables I and II). The chromogens destroyed might be 17-ketosteroidal (ketonic), nonketonic, or both. Until this point is established, the place of the ketonic fraction as the "true" 17-ketosteroid content of the same crude extract is indeed dubious. Nor does one know whether the corrected crude value should agree with the corrected or the uncorrected ketonic result. If there were no destruction in the Girard procedure, the uncorrected ketonic fraction values would be too high, because their colors are less pure than the standard. The corrected value for the ketonic fraction tends to underestimate because of destruction of chromogens. Empirically one could accept a correction of the analysis on the crude extract as reliable if its value were between the corrected and the uncorrected ketonic results.

In summary, the ketonic fraction is not pure, but contains colors and chromogens. It contains 17-ketosteroids whose absorption differs from the standard. Thus its absorption curve is not the same as that of the standard, and the analytical difficulties with the crude extract must also be circumvented with the ketonic fraction. Decomposition occurs in obtaining the ketonic fraction. Thus there is no reason for corrected analyses on the crude extract (Table I, Column 4; Table II, Column 5) to agree perfectly with analyses on the ketonic fraction (Table I, Column 3; Table II, Column 4).

To check our technique and reagents, samples of the standard were submitted to the Girard procedure. Examples of the good recoveries achieved are shown in Table V.

TABLE V. RECOVERIES OF KNOWN AMOUNTS OF STANDARD IN THE GIRARD PROCEDURE

1. ORIGINAL AMOUNT (MG.)	2. KETONIC FRACTION (MG.)		3. NONKETONIC FRACTION (MG.)	
	UNCORRECTED	CORRECTED BY ABSORPTION PEAK	UNCORRECTED	CORRECTED BY ABSORPTION PEAK
1.0	1.0	1.0	0.0	0.0
1.0	1.0	1.0	0.0	0.0

Behavior of Urine Extract Blanks (Intrinsic Color) and Nonketonic Chromogens With Respect to Beer's Law.—The total color intensity measured in the Zimmermann reaction on a crude extract is the sum of the absorbancies of several component colors. The behavior of this total color with respect to Beer's law is determined by the behavior of the absorbancies of the components in this regard. Component colors considered here are (a) urine extract blanks, and (b) nonketonic chromogens. Urine extract blanks (a) are found to follow Beer's law over a wide range of concentrations, confirming a previous observation.[13] Several dilutions of representative crude urine extracts were made up, covering approximately twice the concentration range at which the specimens would be analyzed for 17-ketosteroids. One-half milliliter portions of these dilutions were carried through the entire analytic procedure for urine extract blanks as described under "Methods."* After dilution the corresponding absorbancies were measured at 510 mμ and 425 mμ. These absorbancies, plotted against concentration in Fig. 3, show adherence to Beer's law within experimental error.

Nonketonic chromogens (b), obtained by the Girard separation and subjected to the Zimmermann reaction as these procedures are described in "Methods," were analyzed at two concentrations (Table VI). Amounts taken

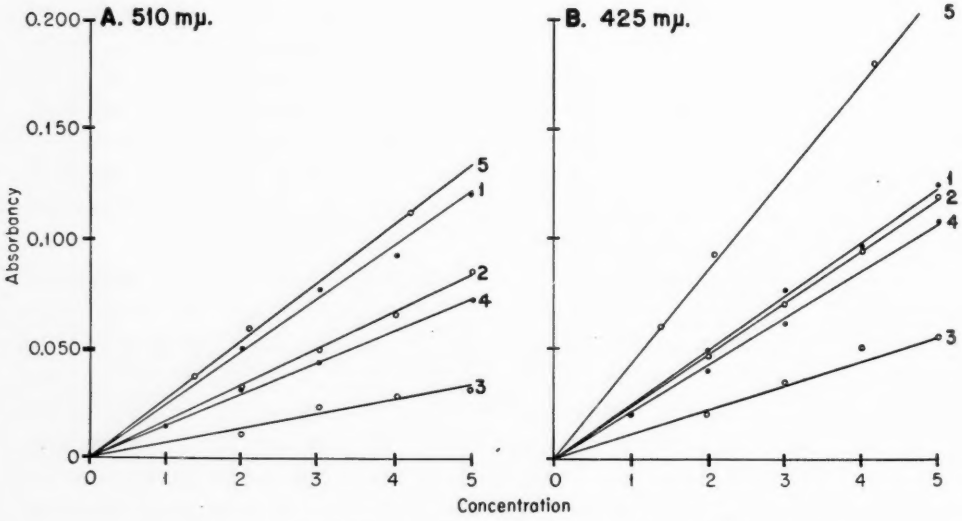
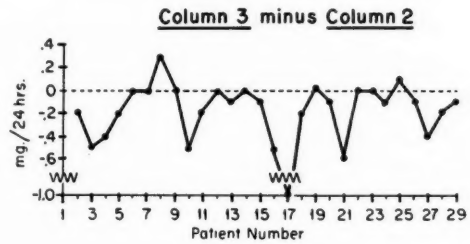


Fig. 3.—Urine extract blanks. Relation of total color to concentration.

*The complete procedure must be followed because of the bleaching of urine extract blanks on incubation with 2.5N alcoholic potassium hydroxide, cf. "Methods."

TABLE VI. NONKETONIC ZIMMERMANN-REACTING CHROMOGENS. RELATION OF TOTAL AMOUNT TO CONCENTRATION OF SPECIMEN BEING ANALYZED (MG./24 HR.; NONKETONIC BLANKS SUBTRACTED)

1. SPECIMEN	2. SAME CONC. AS CRUDE URINE EXTRACT	3. TRIPLE CONC. IN CRUDE URINE EXTRACT	4. COLUMN 2 CORRECTED AS DESCRIBED IN METHODS 2	5. COLUMN 3 CORRECTED AS DESCRIBED IN METHODS 2
1. H. 68	1.0	—	0.0	—
2. H. 68A	0.6	0.4	0.0	0.0
3. H. 70	1.2	0.7	0.0	0.0
4. R. 69	1.3	0.9	0.0	0.0
5. R. 69 (repeat)	0.9	0.7	0.0	0.0
6. R. 69A	0.6	0.6	0.0	0.0
7. R. 71	1.4	1.4	0.0	0.0
8. R. 71 (repeat)	1.5	1.8	0.0	0.0
9. B. 106	0.8	0.8	0.0	0.0
10. B. 117	1.4	0.9	0.0	0.0
11. L. 112	0.7	0.5	0.0	0.0
12. R. 113	0.4	0.4	0.0	0.0
13. K. 110	0.1	0.0	0.0	0.0
14. P. 109	0.1	0.1	0.0	0.0
15. O. W. 80-1	0.9	0.8	0.0	0.0
16. O. W. 80-2	0.8	0.3	0.0	0.0
17. F. K. 83	1.9	0.9	0.0	0.0
18. L. O. 84	1.0	0.8	0.0	0.0
19. G. G. 101	0.0	0.0	0.0	0.0
20. G. G. 105	0.2	0.1	0.0	0.0
21. R. P. 116	1.1	0.5	0.0	0.0
22. B. H. 87	0.4	0.4	0.0	0.0
23. B. H. 97	0.1	0.1	0.0	0.0
24. B. H. 99	0.3	0.2	0.0	0.0
25. A. S. 96	0.1	0.2	0.0	0.0
26. A. S. 100	0.4	0.3	0.0	0.0
27. D. N. 107	0.6	0.2	0.0	0.0
28. J. T. 111	0.7	0.5	0.4	0.0
29. A. S. 119	0.2	0.1	0.0	0.0
	Mean = 0.7	*Mean = 0.5*		



corresponded to what had been present in the same crude extract when analyzed before Girarding (Column 2) and tripling this amount (Column 3). In this way the quantity of (b) taken for analysis was that present when appropriate dilutions of the same crude extract were analyzed before Girarding (ignoring destruction of chromogens in the Girard procedure and possible inclusion of non-17-ketosteroidal chromogens in the ketonic fraction). Table VI, Column 2 shows that an average of 0.7 mg. of Zimmermann-reacting nonketonic chromogens is excreted per 24 hours (expressing the color generated in terms of our standard). Within the limitations of the procedure used, only this much (b) is measured as 17-ketosteroid in uncorrected analyses of crude

extracts. Comparison of Columns 2 and 3 of Table VI shows that (b) is heterogeneous in relations between concentration and absorbancy over this threefold range of concentration. Usually there is negative deviation from Beer's law which will be found in uncorrected crude extract analyses, since the nonketonic fraction is a component of all crude urine extracts. Thus, at higher concentrations, lower 17-ketosteroid values will be found for unknowns, but on the average probably only 0.2 mg. and hence not significant as these determinations are routinely carried out. Failure of nonketonic chromogens to follow Beer's law as closely as the standard gives no appreciable error when uncorrected analyses measured on crude urine extracts at different concentrations are compared. (The nonketonic blank, which follows Beer's law, was subtracted in computing the data of Table VI). But this does demonstrate the necessity for carrying out the determination of ketonic and nonketonic chromogens at the same concentration as that at which the same original 17-ketosteroid extract was analyzed, as was done in demonstrating the destruction of chromogens by the Girard procedure, Table IV.

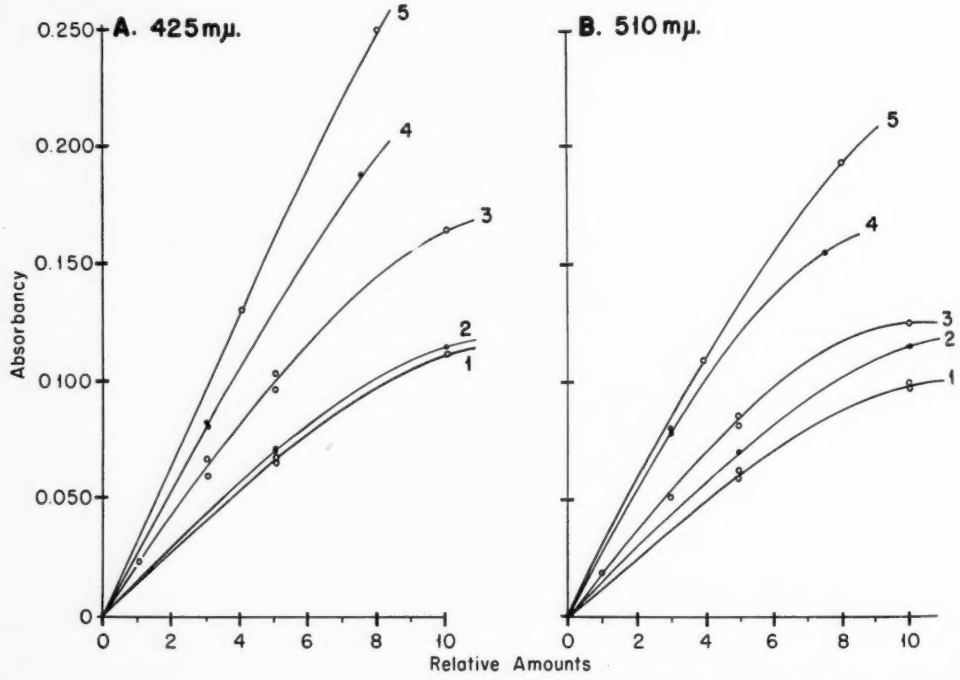


Fig. 4.—Nonketonic Zimmermann-reacting chromogens and blanks. Relation of total color to concentration.

These data show that blanks and nonketonic chromogens were treated correctly in computing data of Tables I and II.

We also checked the behavior of crude urine extracts and their ketonic fractions with respect to Beer's law. Essentially our findings confirm those of many other workers: under Callow-Zimmermann conditions material from urine shows nearly the same relation between absorbancy and concentration as the standard.

This is the premise upon which all of these analyses are based. Data in Tables I, II, and IV have no appreciable error from this source.

Plots of absorbancy against concentration for some of our representative nonketonic fractions (b) not included in Table VI are shown in Fig. 4. Zimmermann reactions were carried out as described previously. Reagent blanks (but *not* nonketonic blanks) were subtracted before plotting the curves. The heterogeneity of these fractions is obvious. Some almost follow Beer's law. The tendency toward negative deviation from Beer's law at 510 and 425 mμ for samples of (b) and their blanks agrees with Table VI. With the Fisher photometer, negative deviations are of greater magnitude. There is a greater tendency for these chromogens to adhere to Beer's law at 425 than at 510 mμ. These findings are significant if correction for pigments and chromogens by correction equations is attempted. In order to put these findings into proper perspective it is necessary to review the application of correction equations to this problem.

Correction Equations.—Because the color developed in urinary 17-ketosteroid extracts by the application of the Zimmermann reaction[10, 14] is not exactly comparable with the color developed in the specific 17-ketosteroid used as the reference standard,[1, 10, 15] compensation for these differences by color correction equations has been advocated and studied by several investigators.[6-8, 10, 16-23] Color correction equations have been expressed in different forms by the several investigators.* All forms are derivable from solution of the following pair of simultaneous equations, in which additive absorbancies[3] are implicit.

$$\begin{aligned} c_a(a_s)_{a\lambda_1} + c_d(a_s)_{d\lambda_1} &= (k_s)_{m\lambda_1} \\ c_a(a_s)_{a\lambda_2} + c_d(a_s)_{d\lambda_2} &= (k_s)_{m\lambda_2} \end{aligned} \qquad (A)$$

The expressions in (A) are applied to this problem as follows: Crude urine extracts are considered as consisting of mixtures of pure 17-ketosteroids and contaminants. If the pure 17-ketosteroid concentration is called c_a, then the concentration of contaminants grouped together as a single entity is c_d:

$(a_s)_{a\lambda_1}$ = absorbancy index of the pure 17-ketosteroids in the green,†
$(a_s)_{d\lambda_1}$ = absorbancy index of the contaminants in the green,
$(a_s)_{a\lambda_2}$ = absorbancy index of pure 17-ketosteroids in the violet,
$(a_s)_{d\lambda_2}$ = absorbancy index of contaminants in the violet,
$(k_s)_{m\lambda_1}$ = absorbancy coefficient of the mixture in the green,
$(k_s)_{m\lambda_2}$ = absorbancy coefficient of the mixture in the violet.

Solving equations (A) simultaneously for the desired c_a gives[3]:

$$c_a = \frac{\dfrac{(k_s)_{m\lambda_1}}{(a_s)_{d\lambda_1}} - \dfrac{(k_s)_{m\lambda_2}}{(a_s)_{d\lambda_2}}}{\dfrac{(a_s)_{a\lambda_1}}{(a_s)_{d\lambda_1}} - \dfrac{(a_s)_{a\lambda_2}}{(a_s)_{d\lambda_2}}} . \qquad (B)$$

*See Reference 7 (p. 230) for the correct form of the equation used in Reference 6.

†Colors (green = absorbancy maximum for 17-ketosteroids and minimum for contaminants; violet = absorbancy minimum for 17-ketosteroids and maximum for contaminants) are used here merely to indicate the spectral regions where absorbancies are measured. More exact data concerning the wave lengths used by various investigators are found in Table VII.

If numerator and denominator of (B) are multiplied by $(a_s)_{d\lambda_1}$, then:

$$c_a = \frac{(k_s)_{m\lambda_1} - \frac{(a_s)_{d\lambda_1}}{(a_s)_{d\lambda_2}} (k_s)_{m\lambda_2}}{(a_s)_{a\lambda_1} - \frac{(a_s)_{d\lambda_1}}{(a_s)_{d\lambda_2}} (a_s)_{a\lambda_2}} \quad . \quad (C)$$

If $\frac{(a_s)_{d\lambda_1}}{(a_s)_{d\lambda_2}} = K$, then a convenient form of (C) may be written as follows:

$$c_a = \frac{(k_s)_{m\lambda_1} - K(k_s)_{m\lambda_2}}{(a_s)_{a\lambda_1} - K(a_s)_{a\lambda_2}} \quad . \quad (D)$$

Expression (D) is used here, and has the same form as the equation in Reference 21: c_a is the amount of 17-ketosteroid in the sample expressed as milligrams of the 17-ketosteroid used as the standard; $(a_s)_{a\lambda_1}$ and $(a_s)_{a\lambda_2}$ are the absorbancy indices of the standard in the green and violet, respectively, under the conditions of the determination; and $(k_s)_{m\lambda_1}$ and $(k_s)_{m\lambda_2}$ are the total absorbancies,* in the green and violet, respectively, of the specimen being analyzed. K is evaluated experimentally as follows: Crude urine extracts are subjected to the Girard separation, and $(a_s)_{d\lambda_1}$ and $(a_s)_{d\lambda_2}$ are measured for the nonketonic fraction at the indicated wave lengths under the conditions of the determination. Only if the nonketonic fraction approximates the contaminants will the K thus obtained be valid. Additive absorbancies (adherence to Beer's law) are assumed in the simultaneous equations (A) from which (D) was derived. But by expressing K in the form of a ratio, the contaminants (the nonketonic component of this mixture) need no longer show additive absorbancies at the indicated wave lengths, as long as the ratios of these absorbancies are constant. The nonketonic fraction cannot readily be determined quantitatively if its absorbancy does not follow Beer's law, but the absorbancy contribution of this component can be corrected for if the ratio K is constant and independent of concentration.[21]

The various correction equations used by the several investigators were converted to the form (D), and all "K's" were expressed in the same terms, so that a direct comparison of numerical values is made in Table VII. Precisely what pigments and chromogens are embodied in K has never been clearly defined. By conversion of the K values of all investigators to the same dimensions (Table VII), it is seen that nearly all K's are of the same order of magnitude. Most of the investigators followed the Callow method, where ordinarily a reagent blank, but no urine extract blank, is run. The available data would then preclude expressing K in any terms other than

$$\frac{(a_s)_{d\lambda_1} - (a_s)_{\text{reagent blank}\lambda_1}\dagger}{(a_s)_{d\lambda_2} - (a_s)_{\text{reagent blank}\lambda_2}}$$

Thus $(a_s)_{d\lambda_1}$ and $(a_s)_{d\lambda_2}$ represent the sums of the absorbancies of the urine extract blanks and nonketonic chromogens at the corresponding wave lengths. The urine extract blank presumably is represented after the Girard separation by the nonketonic (intrinsic color) blank.

*Reagent blanks are always subtracted from absorbancy indices and total absorbancies.

†Reagent blanks should be measured and subtracted if they show appreciable variation from run to run. Unless the reagent blank is only a small percentage of $(a_s)_{a\lambda_1}$ and $(a_s)_{a\lambda_2}$, evaluation of the latter at several different concentrations of standard will give different values for the absorbancy indices of the standard.

TABLE VII. SUMMARY OF CORRECTION EQUATION DATA. ALL K VALUES EXPRESSED IN THE SAME FORM

1. REF.	2. METHOD OF HYDROLYSIS (TIME) AND EXTRACTION	3. METHOD OF ANALYSIS	4. INSTRUMENTS; READINGS; COLOR OR WAVE LENGTH IN Mμ	5. K (RANGE) NO. OF MEASUREMENTS	6. $\frac{(a_s)_{a\lambda_1}}{(a_s)_{a\lambda_2}}$ FOR STANDARD
6	Simultaneous hydrol. with 15 vol. % of conc. HCl, and continuous extn. with CCl_4 at the boiling point[15]	Callow	Evelyn; green, blue	0.5*	2.2
7	Same as 6, but independent hydrolysis and extn.	Callow	Klett-Summerson; 520, 420	0.67 (0.56-0.77) 19	2.5
		Holtorff-Koch	Klett-Summerson; 520, 420	0.67 (0.56-0.77) 8	1.7
17	Combined acid hydrolysis and benzene extn. at the boiling point	Callow	Coleman, Jr.; 530, 470	0.79 15	1.7
18 19 20	10% by vol. of conc. HCl added to boiling urine (10 min.), extn. with CCl_4 at the b.p.	Callow	520, 430	0.6	2.2
21	15 vol. % of conc. HCl added to boiling urine (7 min.), continuous extn. with ether	Holtorff-Koch	Coleman, Jr.; 515, 440	0.77 (0.76-0.82) 6	
8	Three different methods of acid hydrolysis with simultaneous solvent extn.	Callow	Ilford; green, violet	0.83 300 pooled specimens	2.8
22	HCl and neutral hydrolyses at the boiling point and simultaneous benezene extn.	Callow	Klett-Summerson; 520, 420	0.42	2.5
23	15 vol. % conc. HCl (17 min.), ether extn.	Callow	Beckman, Model DU 520, 420	0.5 20 pooled specimens	3.3
	This study	Callow	Beckman; 510, 425	0.71 (0.53-0.96) 61	Mean = 1.90 ± .05 (S.D.)
			Fisher; 525, 425	0.59 (0.43-0.75) 54	Mean = 1.74 ± .07 (S.D.)

*The correction equation in Reference 6 is not in the same form as D, but the K (for chromogens) must be as stated $\frac{E_G}{E_B}\left[\frac{(a_s)_{d\lambda_1}}{(a_s)_{d\lambda_2}}\right]$ because the value given for K of 0.5 [6, p. 212] is compatible only with this form for K, which is the same as in equation D.

In the evaluation of K, several concentrations of the nonketonic fraction from a given specimen are subjected to the Zimmermann reaction. At least one concentration of the nonketonic fraction should be the same as that at which the original crude extract was analyzed. The total resultant absorbancy is usually small in this latter case; e.g., for a urine extract from a normal individual, which typically has a low pigment and chromogen content: at 510 mμ, reagent blank = 0.015; nonketonic intrinsic color blank = 0.040; nonketonic chromogen = 0.040; total = 0.095 absorbancy units. When the reagent blank is subtracted from the total absorbancy, the errors in the measurement of these two small values are added. The total error will be less if the intrinsic color blank is not subtracted, but is included in K. Even though these individual small values are fairly precisely and accurately measured with the Beckman spectrophotometer, accurate measurement cannot be achieved with filter photometers, where low absorbancies are overestimated. Thus, because of the small values of the absorbancies, it is best to attempt to include the urine extract blanks (as the nonketonic color pigment blanks) in K. That such was done appears even more probable when it is recalled that urine extract blanks were considered to be of such small magnitude that they could be ignored, in which case they must of necessity be included in K. Finally, it is advantageous to include both blanks and chromogens in a K value if both can be corrected for simultaneously. In one case[21] where the reagent blank is presumably low, it apparently was not run, so the K obtained must include the three components.

The data of Fig. 4 contraindicate use of a K value in correcting for pigments and chromogens. With increasing concentration the absorbancy of the sum of nonketonic blanks and Zimmermann chromogens rises faster at 425 mμ, $(a_s)_{d\lambda_2}$, than at 510 mμ, $(a_s)_{d\lambda_1}$. This means that the value of the ratio $K = \frac{(a_s)_{d\lambda_1}}{(a_s)_{d\lambda_2}}$ cannot be constant for the variable amounts of nonketonic Zimmermann-reacting chromogens and their blanks in crude urine extracts. The higher their concentration, the lower the value of K. This is seen in Table VIII where K values measured at two different concentrations are tabulated. It is obvious that K's measured at higher concentrations with the Beckman instrument have the lower values (Column 3). A random distribution of magnitudes of K values vs. concentrations is obtained with the Fisher instrument (Columns 4 and 5). In eight cases the higher concentration result is greater, in fourteen cases the lower concentration result is the greater, and in one case both have the same value. Technically, the Fisher instrument was being used here at the upper limit of its powers of discrimination, so no clear-cut result could be obtained. If higher concentrations of the nonketonic fractions were compared, it is probable that there would be closer agreement between values at the different concentrations. However, the nonketonic fractions would then be measured at higher concentrations than were present in the crude extract. The applicability of the results to the problem of correction would then be questionable.

TABLE VIII. EFFECT OF CONCENTRATION OF THE NONKETONIC FRACTION ON THE VALUE OF K

	BECKMAN		FISHER	
1. SPECIMEN	2. K MEASURED AT THE SAME CONCENTRATION AS THE CRUDE URINE EXTRACT	3. K MEASURED AT TRIPLE THE CONCENTRATION IN THE CRUDE URINE EXTRACT	4. K MEASURED AT THE SAME CONCENTRATION AS THE CRUDE URINE EXTRACT	5. K MEASURED AT TRIPLE THE CONCENTRATION IN THE CRUDE URINE EXTRACT
1	0.82	*	0.61	0.60
2	0.83	0.77	0.63	0.60
3	0.86	0.78	0.63	0.60
4	0.70	0.69		0.48
5	0.67	0.61		0.43
6	0.71	0.70		0.59
7	0.85	0.77		0.68
8	0.55	0.63		
9		0.82		0.70
10	0.59	0.56	0.49	0.53
11	0.64	0.66		0.67
12		0.61		0.50
13	0.59	0.54	0.43	0.49
14	0.59	0.56	0.50	0.50
15	0.71	0.66	0.44	0.56
16	0.72	0.72	0.61	0.64
17	0.85	0.78	0.71	0.74
18	0.67	0.67	0.55	0.63
19	0.59	0.53	0.45	0.44
20	0.69	0.67	0.59	0.58
21	0.75	0.70	0.67	0.66
22	0.75	0.76		0.69
23	0.83	0.77	0.64	0.66
24	0.96	0.89	0.74	0.69
25	0.86	0.79	0.71	0.61
26	0.74	0.68	0.62	0.50
27	0.70	0.64	0.67	0.49
28	0.64	0.58	0.51	0.50
29	0.75	0.70	0.62	0.58
30	0.94	0.84	0.75	0.68
31	0.68	0.59	0.42	0.44
32	0.76	0.68	0.66	0.59
	Mean = *0.71*		*Mean* = *0.59*	
	Standard deviation = *0.10*		*Standard deviation* = *0.09*	

*Wherever data are omitted, a reliable result was not obtained at that concentration. In the case of Fisher data this was usually because the concentration which had to be used gave absorbancies too low to be measured reliably.

Comparison of K values determined in the course of this investigation with those of other workers (Table VII) shows the degree of correlation that might be anticipated. Since the mean K value is a function of the colorimeter, the transmission curves of the filters employed, as well as the concentration and number and diversity of the samples studied, we got the expected results with the Beckman and Fisher instruments. The magnitude of the standard deviations of the mean K values suggests too great a variability in K's for their practical use.

The extensive alterations in the color of the urine extract blank caused by the Girard procedure has been shown in Table III. Alterations in these colors that change their ratio in the green and violet affect the value of K, and would be a source of error in correction by equation. So an additional assumption must be made, that, although the colors are altered, such changes as occur in the green and violet regions of the spectrum do not alter the K

ratios. Since *all* of the urine extract blank was included in the measurement of the crude extract value, all of the urine extract blank color should be included in K, but some has been lost in evaluating K, so an error has probably been introduced.

Ketonic fractions are also colored (i.e., ketonic blank). This coloring may contribute significant absorbancy in the case of pathologic urines, and almost always there is absorbancy even with normal urines. Since the color in the ketonic blank was also in the orginal urine extract blank, it should be included in K. The absence of the ketonic blank colors will introduce a further error, unless the ratio of their absorbencies in the green and violet are the same as the K computed from the nonketonic fraction. These pigments are partly responsible for the fact that a single Girard separation often does not give a pure ketonic fraction.

Destruction of chromogens by the Girard procedure has been shown in Table IV.

Therefore the Girard procedure is inadequate for obtaining a K which corrects for contaminants: (1) by failing to localize all pigments and chromogens in the nonketonic fraction, and (2) by partly destroying them.

The data on K values were used to compute the results of Table IX as follows:

1. Columns 2a, 4a, and 3 and 5 were calculated as in "Methods," 1 and 3, respectively.
2. The 17-ketosteroid content corrected by equation D:
   a. 1a from "Methods," 1.
   b. $(A_s)_{\text{standard 425}} - (A_s)_{\text{reagent blank 425}} =$ value for plotting standard curve at 425 mμ.
   c. $(A_s)_{\text{17-ketosteroid 510}} - (A_s)_{\text{reagent blank 510}} = (k_s)_{m\lambda_1}$.
   d. $(A_s)_{\text{17-ketosteroid 425}} - (A_s)_{\text{reagent blank 425}} = (k_s)_{m\lambda_2}$.
   e. Equation D: $(a_s)_{a\lambda_1}$ and $(a_s)_{a\lambda_2}$ were determined by the slopes of the corresponding standard curves at 510 and 425 mμ, respectively; K was evaluated experimentally; $(k_s)_{m\lambda_1}$ is 2c and $(k_s)_{m\lambda_2}$ is 2d. Substitution of this in equation D gives the amount of 17-ketosteroid taken for analysis. Corrected values were computed using their own K's (Columns 2b and 4b) and the respective mean K's (Columns 2c and 4c).

Table IX shows that there is often agreement between ketonic fraction values (Columns 3 and 5) and data corrected by equation using K values (Columns 2 and 4, b and c). This conforms with findings of other workers.[6-8] But the inconsistencies expected because K cannot be correctly evaluated are also present. This is especially true of the Fisher instrument even though the filters were selected because on measurement they showed narrow transmission bands at the appropriate wave lengths. Gross disagreement between uncorrected ketonic fraction values and crude extract data corrected by equations has also been shown by other workers.* Perfect agreement was not achieved

*Reference 24, Table 2, p. 100.

TABLE IX. COMPARISON OF 17-KETOSTEROID ANALYSES OBTAINED BY THE CORRECTION EQUATION

1. PERSON	BECKMAN				FISHER			
	2. CRUDE URINE EXTRACT			3.	4. CRUDE URINE EXTRACT			5.
	A. UNCORRECTED	B. CORRECTED BY OWN K	C. CORRECTED BY OUR AVERAGE K	GIRARDED KETONIC FRACTION (UNCORRECTED)	A. UNCORRECTED	B. CORRECTED BY OWN K	C. CORRECTED BY OUR AVERAGE K	GIRARDED KETONIC FRACTION (UNCORRECTED)
H. 68	13.5	13.4	16.7	12.7	14.5	13.7	14.0	11.7
H. 68A	15.6	14.5	15.3	12.1	14.9	15.7	16.4	11.3
H. 70	19.0	18.2	18.2	16.2				
R. 69	21.5	18.3	18.8	19.8	19.8	16.8	17.5	19.8
R. 69A	24.0	20.7	20.7	20.5	22.1	21.3	21.9	19.7
R. 71	31.1	27.9	25.1	27.7	29.9	22.2	25.7	24.4
B. 106	29.7	28.6	29.1	27.3	28.6	26.7	27.4	24.6
B. 117	28.1	25.5	26.5	21.9	27.6	26.1	26.8	21.8
L. 112	19.1	18.9	18.9	18.6				
R. 113	9.6	9.2	8.9	8.1	9.6	9.0	8.9	7.6
J. II-28	8.7		8.6	7.0	8.5		9.9	6.8
F. II-32	9.4		8.6	7.4	9.5		10.1	7.8
K. 110	1.4	1.6	1.3	1.2	1.3	1.5	1.2	1.2
P. 109	1.1	0.9	1.0	0.9	1.0	0.9	0.9	0.8
O. W. 80-1	7.6	5.6	5.9	5.0	7.1	5.0	6.1	5.1
O. W. 80-2	6.9	4.0	5.6	5.1	6.4	2.9	5.6	4.8
R. K. II-20	3.8		2.8	1.8	3.9		5.2	2.1
R. K. II-24	3.4		2.3	2.9	3.4		5.9	3.4
J. C. II-22	3.5		4.5	2.9	3.7			2.8
F. K. 83	9.3	7.8	3.9	6.1	7.2	7.3	4.2	5.3
L. O. 84	2.9	1.9	1.8	3.0	2.8	2.3	1.8	1.8
G. G. 88	1.1	0.3	0.5	0.7				
G. G. 101	1.0	1.0	0.8	1.0	1.0	0.9	0.9	1.0
G. G. 105	2.3	1.4	0.7	2.0	2.1	1.4	0.9	2.0
R. P. 116	21.6	22.4	23.6	18.0	20.7	22.0	22.6	16.9
B. H. 87	2.9	1.9	1.6	1.9	2.5	1.4	1.8	1.4
B. H. 99	6.1	5.9	6.0	6.2	5.8	5.2	5.6	5.6
A. S. 96	3.7	3.0	2.8	3.0	3.6	3.1	3.1	2.9
A. S. 100	4.0	3.1	2.4	2.8	4.1	3.5	2.8	2.7
N. C. II-34	2.0		1.6	1.2	2.0		2.5	1.4
L. L. I-138	2.1		0.8	1.1	2.3		2.1	1.2
D. N. 107	15.3	15.5	17.2	14.3	14.0	14.1	15.3	13.0
A. S. 119	1.4	1.2	1.2	1.1	1.5	1.1	1.3	1.0

between corresponding uncorrected results from the two instruments (cf. Columns 2a with 4a, and 3 with 5) despite special efforts to run analyses at optimal concentrations.

Reagent blanks are measured whether correction is by equation or absorption peak. They average 0.030 absorbancy units (or optical density units) at 510 mμ and show varying absorption spectra. Usually there is higher absorbancy at 425 than at 510 mμ, but occasionally both are the same.[25] Subtraction eliminates the contribution of the reagent blank when correcting by equation. With the absorption peak method the contribution of the reagent blank to the peak is corrected for by "Methods," 2a.

Intrinsic colors (blanks) and chromogens are grouped together and corrected for as a unit when using the correction equation. In correction by absorption peak, colors and chromogens are corrected for individually. The colors are measured as the urine extract blank. Their absorption spectra are extremely variable. They do not show linear absorbancy from 425 to 595 mμ. This deviation from linear absorbancy is corrected by "Methods," 2c.

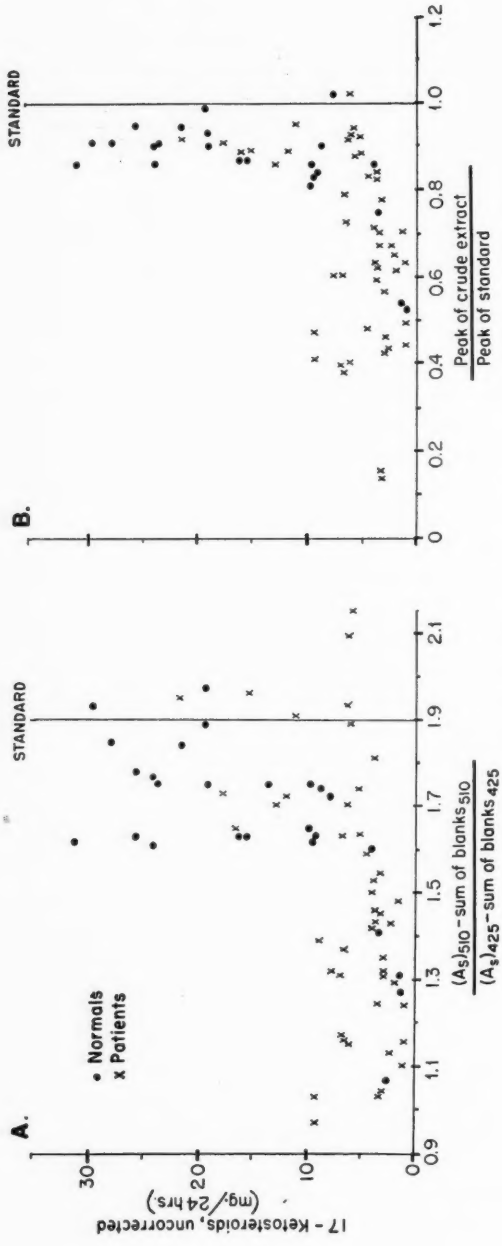


Fig. 5.—Color comparisons of standard and crude extracts.

Correction by equation assumes colors *and* chromogens to show a constant ratio between absorbancies measured at the two selected wave lengths. As we have seen, this is not true (Table VIII). Correction by absorption peak as used in this paper assumes only that chromogens show linear absorption between 425 and 595 mμ. Calculation procedure 2 in "Methods" corrects for all chromogens showing linear absorbancy between 425 and 595 mμ regardless of variations in their slope.

Correction by equation also assumes that a true K value can be obtained from the nonketonic fraction. This is not required when correcting by absorption peak. Data on the nonketonic fraction in Table VI can be used to show that the chromogens (only a portion of the total) obtained by Girard separation do show linear absorbancy from 425 to 595 mμ. With measurements at three wave lengths and calculation as in "Methods" 2, the colored portion of this fraction is corrected for by 2c (using the nonketonic blank). The chromogens then have the value of zero (Columns 4 and 5), although the amounts in Columns 1 and 2 are present by measurement at one wave length. The nonketonic fraction is an artificial one, not telling what pigments and chromogens were really present in the urine extract. However, those chromogens which are still present do show linear absorbancy from 425 to 595 mμ.

The superiority of corrected analyses on the crude extract and the possibility for error in uncorrected analyses on ketonic fractions can be seen from Table II. In specimens R. K. II-20 and II-24, even after Girard separation, only about one-half of the chromogens in the ketonic fraction show a peak at 510 mμ (cf. Column 4, corrected vs. uncorrected). Data from direct correction of the crude extract value (Column 5) are low, but are a better estimate of 17-ketosteroidal chromogen content than the uncorrected result on the ketonic fraction. O. W. 80-1 and 80-2 are specimens where only about two-thirds of the crude extract (compare Column 3 with 5) and three-fourths of the ketonic fraction (Column 4, compare corrected with uncorrected) are 17-ketosteroidal chromogens. Here too, the uncorrected ketonic fraction analysis overestimates and the best result is by correcting the analysis on the crude extract. Data of B. H. show excretion before, 87, and after, 99, cortisone therapy. When uncorrected crude extract values are compared, one is uncertain whether there is a significant change in excretion on therapy. The uncorrected ketonic fraction value from 87 suggests that two-thirds of the chromogens in the same crude extract are 17-ketosteroidal. But only one-half of this ketonic fraction (or one-third of the same crude extract value) is 17-ketosteroidal chromogen (Column 4, corrected). On cortisone therapy all of the chromogens in the crude extract give a peak at 510 mμ (cf. Columns 3 and 5 of B. H. 99). The ketonic fraction is more chromogenic at 510 mμ than the standard and gives a higher peak (cf. Column 4, uncorrected and corrected). This difference in 17-ketosteroidal chromogen content between B. H. 87 and 99 is most easily demonstrated by Column 5. Correction of the crude extract analysis gives a more reliable result than uncorrected analysis of the ketonic fraction.

Throughout this paper we have been representing the relative spectral purity of Zimmermann reaction colors numerically as the relation between uncorrected and corrected data on the same specimen. Color purities may be more concretely pictured as in Fig. 5, *A*. Uncorrected 17-ketosteroid values of the crude extracts are plotted against the ratio of their absorbencies at 510 ("Methods," 1b) and 425 mμ, after subtracting the sum of the reagent and urine extract blanks of the same specimens at corresponding wave lengths. This ratio for the standard (only reagent blanks subtracted): Mean = 1.90; standard deviation of the mean = ± 0.05. If the 24-hour excretion of 17-ketosteroids is greater than 10 mg., colors are comparable to the standard. When the 24-hour output is less than 10 mg., colors may be comparable to the standard, but they tend to be much poorer.

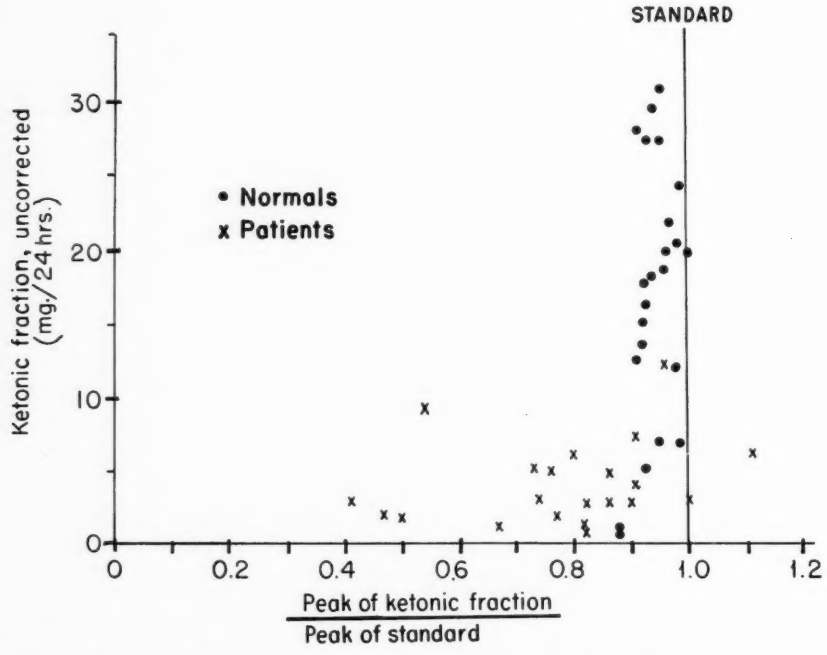


Fig. 6.—Color comparisons of standard and ketonic fractions.

Since three wave lengths are used in our experiments, the peak heights of the unknowns can be compared with the standards as in Fig. 5, *B*. For each extract the ratio of the peak height XY (cf. Fig. 2, *B*) to total absorption XO is divided by the same ratio for the standard (XZ/XO). Reagent blanks are subtracted for the standards and the sums of reagent blanks and urine extract blanks are subtracted for the extracts (as in "Methods," 2). The data show the same relation as Fig. 5, *A*. Urine extracts containing 17-ketosteroids giving high peaks may be found with low or high 24-hour excretion. There is a random distribution of peak heights if the excretion is 10 mg. or less. It is apparent that uncorrected 17-ketosteroid analyses on such specimens may show large errors. Random distribution of peak height of crude extracts is to be expected

because of the great qualitative and quantitative variation in 17-ketosteroid excretion. Apparently there is sufficient high peak material in crude extracts containing more than 10 mg. to give a peak approximating the standard. With low excretions a larger portion of the 24-hour specimen must be analyzed to get a good concentration of 17-ketosteroid. Thus a higher proportion of pigments and chromogens is included in such analyses than when specimens containing more 17-ketosteroids are run at greater dilutions. This apparently does not interfere with detection of high peak specimens containing little total 17-ketosteroid.

Fig. 6 is the same type of plot as Fig. 5, *B*, but for the ketonic fraction. There is a significant difference between the colors of the standard and the ketonic fractions. The absorption spectra of ketonic fractions are more like those of the standard than are the spectra of the crude extracts. The variability in peak height shown by ketonic fractions is consistent with observations on pure 17-ketosteroids.[4, 5] Many 17-ketosteroids give a lower peak than the standard used here, but some are more chromogenic than the standard and give higher peaks, as does B. H. 99, Table II.

SUMMARY

1. Only if a method of color correction is used can the 17-ketosteroid content of a urine extract be expressed in terms of the standard because the two have different absorption spectra in the Zimmermann reaction.

2. The most reliable analysis of a crude extract is obtained by correction, assuming that non-17-ketosteroidal chromogens absorb linearly between 425 and 595 mμ.

3. Uniformly reliable results are not obtained by correction equations because the value of the "constant" (a) shows too great variability with different specimens, (b) is a function of the concentration, and (c) is not representative of what should be corrected.

4. Uncorrected analyses on ketonic fractions may be misleading because (a) Zimmermann reaction absorption spectra of ketonic substances are not the same as the standard and (b) destruction of chromogenic material occurs in the Girard procedure.

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THE EXTRACTION OF AMINO ACID-CONTAINING SUBSTANCES FROM URINE

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WE HAVE reported that acetone solutions of certain organic acids are capable of dissolving amino acids and that the amino acids may be precipitated from these solutions on neutralization with ammonia or some other base whose salt with the acid used is soluble in acetone.[1, 2] The per cent of recovery of amino acids from such solutions varies with the acid, base, and amino acid used. For instance, the recovery of amino acids from alkyl acid phosphate-acetone solutions which have been neutralized with triethylamine is poor. We have also reported on the use of acetone solutions of the organic acids for the preparation of protein hydrolysates free from inorganic contaminants.[3] Pollack and Eades[4] have reported the use of dl-camphorsulfonic acid in acetone for the extraction of glucuronic acid conjugates of aspartic and glutamic acids from urine.

For this present study we have chosen to compare the properties of acetone solutions of trichloroacetic acid (TCA), benzenesulfonic acid (BSA), and dl-camphorsulfonic acid (CSA) in the extraction of amino acids, either free or combined, from urine. We chose these because they had shown promise in a preliminary study and because they are easily available. At the outset, however, certain advantages and disadvantages of each one were apparent. Dr. A. H. Corwin pointed out that in water solution, TCA progressively destroys histidine.[5] We tested this property in acetone solutions and found 25 per cent destruction of histidine occurs if the TCA-acetone-histidine solution is neutralized immediately after its formation and about 50 per cent destruction if the neutralization is delayed for one week. We also found that histidine is hard to dissolve in BSA-acetone solutions. Tryptophan is recovered in negligible amounts when its solution in BSA and acetone is neutralized with ammonia or with triethylamine. CSA-acetone appears satisfactory for the solution and recovery of histidine. If tryptophan alone is dissolved in the minimum amount of this reagent necessary for its solution, recovery is 80 per cent on neutralization with ammonia. However, if a large excess of reagent is used or if the tryptophan occurs in a mixture of amino acids and peptides as in an enzyme hydrolysate, then the precipitation with ammonia results in a low yield of the amino acid. The ammonium salt of CSA is harder to dissolve in acetone than are the ammonium salts of the other two acids. Its limit of solubility on overnight standing in ammoniacal acetone is about 0.2 N. In practice we have found it advisable to dilute CSA-acetone solutions to 0.1 to 0.2 N with acetone before the ammonia neutralization and to allow the precipitates so formed to

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settle overnight before filtering. If this is not practicable, or if any contamination of the precipitates with the ammonium salt of the reagent persists, the offending salt may be washed away from the rest of the solids with ammonia-acetone solution. The ammonium salt of BSA also requires ammoniacal acetone for complete solubility. Hippuric acid is soluble in acetone to the extent of at least 6 per cent. It, therefore, will not be found in the ammonia-precipitated material from acetone solutions of the organic acids.

MATERIALS AND METHODS

All the reagents used in this study were C. P. grade and no further purification of any of them was carried out. The ammonia in acetone solution used was prepared by bubbling anhydrous ammonia into acetone until a saturated solution resulted.

The urine sample used in this study was obtained for us by Dr. Frederick W. Barnes, Jr., of the Johns Hopkins Hospital and Medical School. It was a 48-hour sample which had been evaporated and then stored under acetone. It was reported to contain an abnormally high content of amino acid materials. Upon receipt in our laboratory the urine was diluted to a volume of 400 ml. Samples of 100 ml. were taken, the pH adjusted to about 6 with dilute HCl, and the solutions evaporated in a stream of air on a water bath maintained at a temperature of 90° C. The evaporation was discontinued when four hours' additional heating made no appreciable difference in the dryness of the solids. The evaporated residues were stored under acetone in the refrigerator.

Extraction With TCA-Acetone.—After the acetone was decanted from one of the samples the solids were extracted with a 0.9 N solution of TCA in acetone. Twenty milliliter portions of the acid solution were applied to the solids contained in an evaporating dish, the mixture stirred for one or two minutes, and the extract decanted through Whatman No. 4 filter paper. The first 60 ml. of extract were combined and called Fraction 1. Fractions 2 and 3 were made up of the next 40 and 60ml. of extract, respectively. As the fractionation progressed, the undissolved material became more granular; 14.5 Gm. remained undissolved. This material gave a positive test for amino acid-containing material with ninhydrin.

Extraction With BSA-Acetone.—The extraction with BSA in acetone was carried out on a similar sample in much the same manner. However, as the fractionation progressed, a semisolid substance, insoluble in the reagent, formed. This clogged the pores of the filter paper, and it became necessary to separate the supernatant and undissolved materials by means of a centrifuge. To prepare Fraction 1, 120 ml. of a 0.5 N solution of BSA in acetone were used; 160 ml. each were used for Fractions 2 and 3. A total of 13.8 Gm. remained undissolved. This material gave a negative test with ninhydrin.

Extraction With CSA-Acetone.—The extraction with CSA in acetone was carried out on a similar sample in the same way. Again it was necessary to use a centrifuge for the separation of the supernatants and undissolved materials. Three fractions, each using 160 ml. of a 0.5 N CSA-acetone solution, were prepared; 9.3 Gm. of ninhydrin-negative material remained undissolved.

Precipitation, Reprecipitation, and Analysis of Extracts.—After the CSA in acetone fractions had been diluted with an equal volume of acetone, all the extracts were neutralized with an acetone-ammonia solution. The CSA-acetone extracts were then further diluted with acetone to make the final volume about three times the original one. The precipitates resulting from the neutralization were allowed to settle overnight and were then removed by filtration, washed well with acetone, dried at 80° C., and weighed. The filtrates were tested for amino acid-materials with ninhydrin. Those from the TCA-acetone and CSA-acetone extractions were negative; that from the BSA-acetone, positive. The ammonia precipitated materials from the fractions were then analyzed for amino nitrogen, both before and after hydrolysis with 6 N HCl at 100° C. for 24 hours. These analyses revealed heavy contamination of

the precipitates with nonamino acid materials. Each precipitate, therefore, was redissolved in the reagent used in its preparation and then reprecipitated. In redissolving the original ammonia-precipitated materials, those prepared with BSA or CSA in acetone completely redissolved. However, a precipitate appeared in each of these solutions on standing. These precipitates were removed after being allowed to settle overnight and were tested with ninhydrin. A negative test for amino acid material was obtained in each case. On redissolving the first two ammonia-precipitated fractions from the TCA-acetone extraction, complete solubility did not occur although 1½ to 2 times the volume of solution used to dissolve them originally was used. These undissolved residues were ninhydrin positive and were saved for analysis along with the other fractions.

Further Extraction With TCA-Acetone.—A fourth TCA-acetone fraction was formed by extracting the material left undissolved by the original TCA-acetone fractionation with an additional 50 ml. of the acid reagent. This extract was neutralized as the others had been and the resulting precipitate analyzed along with the others without any further reprecipitation. The material insoluble in TCA-acetone now weighed 11.5 Gm. and was ninhydrin positive.

Further Investigation of the CSA-Acetone-Treated Portion.—The CSA in acetone-treated portions of the urine were subjected to additional tests. Portions of the filtrates from the ammonia neutralization of the original extracts were evaporated and then boiled with 6 N HCl for 3 hours. They now gave a positive test with ninhydrin. Chromatography showed glycine as the only amino acid present, leading to the conclusion that the unprecipitated amino acid material was hippuric acid.

The material undissolved in CSA-acetone solution was refluxed for 5 hours with 6 N HCl. It now gave a positive test with ninhydrin and, after neutralization with ammonia and evaporation to dryness, it was extracted with 60 ml. of a 0.5 N solution of CSA in acetone. On neutralization with ammonia 37 mg. of amino acid material were precipitated. Chromatography of this precipitate showed several amino acid spots indicating that the original material was probably a protein or large peptide. No further analysis was done on this fraction.

Analyses of Fractions.—All the ammonia-precipitated materials were weighed and analyzed for creatinine by the Folin colorimetric method[6] and for amino nitrogen both before and after hydrolysis with 6 N HCl for 24 hours at 100° C. The Folin colorimetric method as modified by Frame, Russell, and Wilhelmi[7] and by Russell[8] was used.

RESULTS

The results of the creatinine and amino nitrogen analyses are summarized in Table I.

TABLE I. THE AMINO NITROGEN AND CREATININE CONTENT OF AMMONIA-PRECIPITATED FRACTIONS FROM ORGANIC ACID-ACETONE EXTRACTS OF URINE

DESCRIPTION OF FRACTION		ORIG. WEIGHT (GRAMS)	WT. AFTER RE-PPTN. (GRAMS)	CREATININE CONTENT (MG.)	AMINO-NITROGEN		
					ORIG. (MG.)	AFTER HYD. (MG.)	RATIO (ORIG: HYD.)
TCA preparations	1	4.15	3.2	134	9.60	19.2	0.50
			ins. 0.5	5.5	1.47	6.4	0.23
	2	3.50	1.28	36	5.12	18.0	0.28
			ins. 0.61	12.2	1.22	3.7	0.33
	3	2.10	1.16	20	6.95	30.1	0.23
	4	0.53	—	5	3.18	9.6	0.33
BSA preparations	1	0.97	0.26	6.5	3.65	13.0	0.28
	2	1.57	0.77	6.8	6.29	15.4	0.41
	3	3.15	1.09	4.3	19.7	21.8	0.90
CSA preparations	1	0.88	0.57	6.8	9.30	15.4	0.59
	2	1.31	0.68	11.0	12.3	27.2	0.45
	3	2.15	1.65	17.3	19.9	29.7	0.67

DISCUSSION

Table I shows that fractions prepared with TCA in acetone contained the most creatinine and also the most nonamino acid material. The ratio of 6.25 times the amino nitrogen after hydrolysis to the weight of precipitated material from the TCA-acetone solutions is 0.08. For the fractions prepared by means of BSA-acetone, this ratio is 0.15; and for the CSA-acetone fractions it is 0.16. The materials which are extracted and recovered on ammonia precipitation by these reagents aside from amino acids and peptides are probably largely organic molecules with which the amino acids are in combination. Urea is not a contaminant due to its large solubility in acetone.

That TCA in acetone is not as efficient a solvent for amino acid materials occurring in urine as are the other two reagents tested is shown by the positive ninhydrin test given by the material undissolved by this reagent, without any hydrolysis. The positive ninhydrin test obtained on the filtrate from the ammonia-neutralized BSA-acetone extracts indicates that appreciable amounts of amino acid materials may be unrecoverable from such filtrates. In this particular urine studied, hippuric acid was the only amino acid-containing compound which was unprecipitated by ammonia from CSA-acetone solution. In any application of this method to other urines, however, it would be well to test the filtrates from such precipitations for amino acid materials both before and after hydrolysis. By hydrolysis of the undissolved material and its subsequent neutralization with ammonia followed by CSA-acetone extraction of the dried solids, the amino acids occurring in combinations which are insoluble in CSA-acetone, such as proteins or large peptides, can be recovered. Hydrolysis of CSA-prepared fractions of the unhydrolyzed urine followed by another CSA-acetone extraction should produce amino acid mixtures which are largely free of contaminants, if this is desired.

SUMMARY

By extracting unhydrolyzed urine solids with CSA solutions in acetone and by subsequently neutralizing these extracts with ammonia, fractions may be obtained which contain the amino acids, either free or combined, in an environment much simpler than that in which they originally occurred in the urine. It is hoped that the use of this method will greatly facilitate the study of amino acid excretion.

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DIFFERENTIAL DIAGNOSIS OF ADRENAL LESIONS BY THE USE OF THE INTRAVENOUS ADMINISTRATION OF HYDROCORTISONE

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THE demonstration by Wilkins and associates[1] that the excessive steroid production in adrenal hyperplasia with virilism can be reduced by the administration of cortisone has led to a new era in the therapy of adrenal hyperplasia with virilism. It has now become possible to convert these virilized women into normal-appearing, potentially fertile females. The demonstration of pregnanetriol as the characteristic urinary steroid found in adrenal hyperplasia with virilism,[2] coupled with other studies, has led to the belief that the basic defect in this disease is a relative inability of the adrenal to hydroxylate a C-21 steroid at the 11 β and 21 positions (Fig. 1). This leads to the production in large measure of an incomplete adrenal steroid, probably 17-hydroxyprogesterone in preponderance.[3]

The compound 17-hydroxyprogesterone is of known natural occurrence and of known androgenicity.[4] It has also been demonstrated to lead to an increased secretion of 17-ketosteroids when administered to normal man.[3] We are not aware as yet of a demonstration that its administration will lead to an increased urinary pregnanetriol excretion. It should be pointed out, however, that the only patients who should be expected to respond to this steroid with an increased pregnanetriol excretion are patients with adrenal hyperplasia with virilism, or patients without adrenals, since people with normal intact adrenals would handle this compound by hydroxylation at the 11β and 21 positions, leading to the excretion or utilization of the normal cortical steroid.

It is believed that the 17-hydroxyprogesterone,[3] unlike normal adrenal cortical hormone, fails to inhibit pituitary ACTH production to an adequate extent, thus allowing the known increased circulating ACTH[5] and resultant hyperplasia. With the increased size of the adrenal resulting from ACTH overproduction, the production of inhibitory steroids eventually attains a sufficient level to inhibit the pituitary to a steady state. This state is one which is greater than normal with respect to the production of pituitary ACTH.

Fig. 2 illustrates our present conception of the balance between the pituitary and the adrenal in the normal individual. The pituitary produces ACTH, which in turn stimulates the production of Compounds F (hydrocortisone) and B by the normal adrenal. These compounds serve as a brake on excessive

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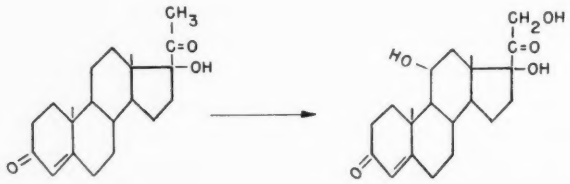
production of ACTH by the pituitary. If the adrenals are removed, this brake is also removed, and ACTH production increases. If the pituitary is removed, ACTH production, of course, will cease, and the adrenal maintains a much-diminished production of Compounds F and B.

Fig. 3 compares the normal situation with the one thought by us to exist in adrenal hyperplasia with virilism. Since the major product of the adrenals is 17-hydroxyprogesterone, and since this does not inhibit the pituitary release of ACTH, a secondary increase in ACTH production and circulation[5] will result with the consequent production of adrenal hyperplasia. Following this,



17-HYDROXYPROGESTRONE (Androgenic) 17-HYDROXYCORTICOSTERONE (Compound F)

Fig. 1.—Structural formula of 17-hydroxyprogesterone and 17-hydroxycorticosterone.

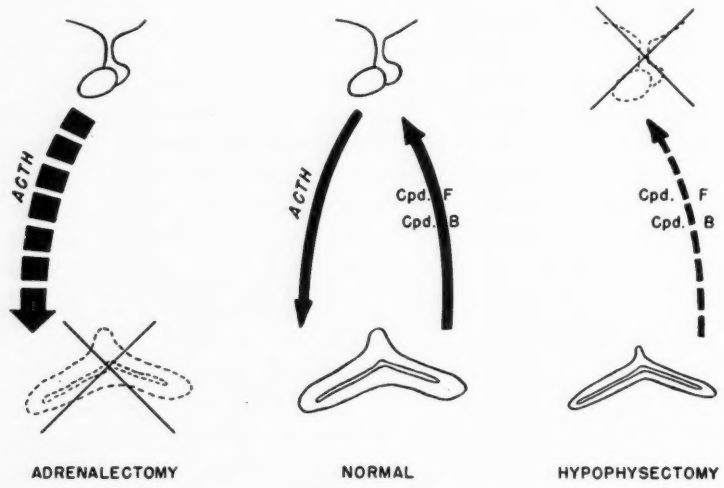


Fig. 2.—Adrenal pituitary axis—normal and as altered by removal of adrenals or pituitary gland.

a greater production of the incomplete steroids, generally believed to be 17-hydroxyprogesterone, occurs. The situation in Cushing's disease with adrenal hyperplasia appears to result from the primary production of excessive ACTH from the pituitary. In this disease, apparently, the pituitary production of ACTH is not inhibited by normal amounts of Compounds F and B in the blood. This leads to excessive production of a normal adrenal cortical hormone secondary to the overproduction of ACTH by the pituitary. In other words, the pituitary's thermostat for ACTH release seems to be set at too high a level.

Fig. 4 illustrates the condition found in adrenal tumors causing virilism or Cushing's syndrome. The virilizing tumors usually produce steroids which do not inhibit the pituitary secretion of ACTH. These androgens are not shown in Fig. 4 because they do not affect the ACTH secretion. As the secretion of Compounds F and B is normal, therefore, the contralateral adrenal is of normal size and function. However, the tumor producing Compounds F and B, resulting in Cushing's syndrome, does inhibit the output of ACTH and leads to the development of atrophy of the residual adrenal tissue on the same side as the tumor, as well as in the contralateral adrenal. This necessitates supportive therapy at the time the tumors associated with Cushing's disease are removed.

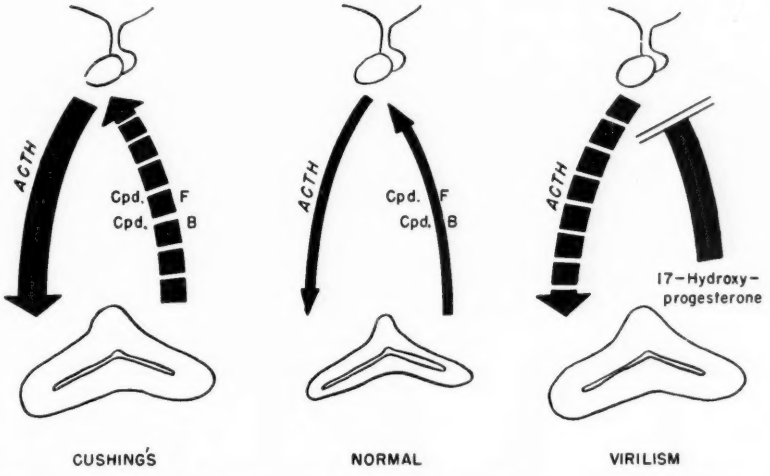


Fig. 3.—Adrenal pituitary axis—as seen in adrenal hyperplasia producing Cushing's disease or virilism.

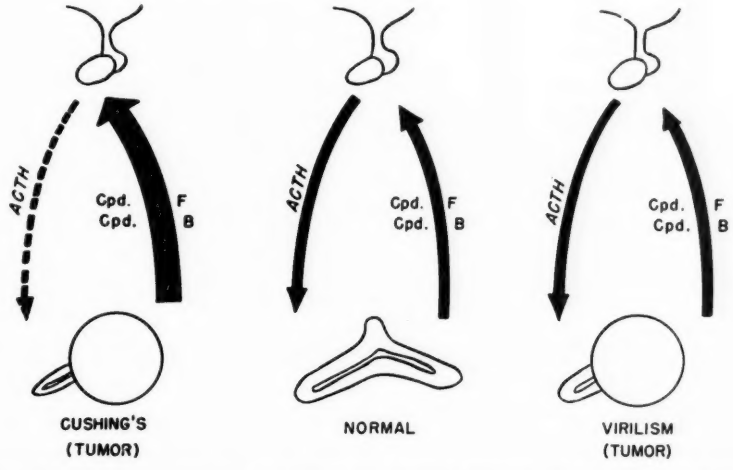


Fig. 4.—Adrenal pituitary axis—as seen in adrenal tumors producing Cushing's syndrome or virilism. The androgenic steroids produced by the virilizing tumor are not shown since they do not affect the adrenal pituitary axis.

Fig. 5 illustrates what is believed to be accomplished by the administration of intravenous hydrocortisone in adrenal hyperplasia with virilism. The effect of ACTH administration in this condition is also shown. The situation in the center illustrates the disease state where the production primarily of 17-hydroxyprogesterone is unable to inhibit the pituitary's release of ACTH so that a secondary increase in circulating ACTH occurs. The 17-hydroxyprogesterone, however, is metabolized to the abnormal end products found in the urine as pregnanediol chromogen. If Compound F is administered in adequate amounts to inhibit the pituitary's production of ACTH, then the stimulus to adrenal steroidogenesis is removed. The 17-hydroxyprogesterone production either ceases or drops to very low levels, and the abnormal steroids, both 17-ketosteroid and pregnanediol chromogen, disappear from the urine. In the opposite situation, where additional exogenous ACTH is administered, the hyperplastic adrenal responds with an additional production of an increased amount of 17-hydroxyprogesterone, leading in turn to an increase in urinary pregnanediol chromogen.

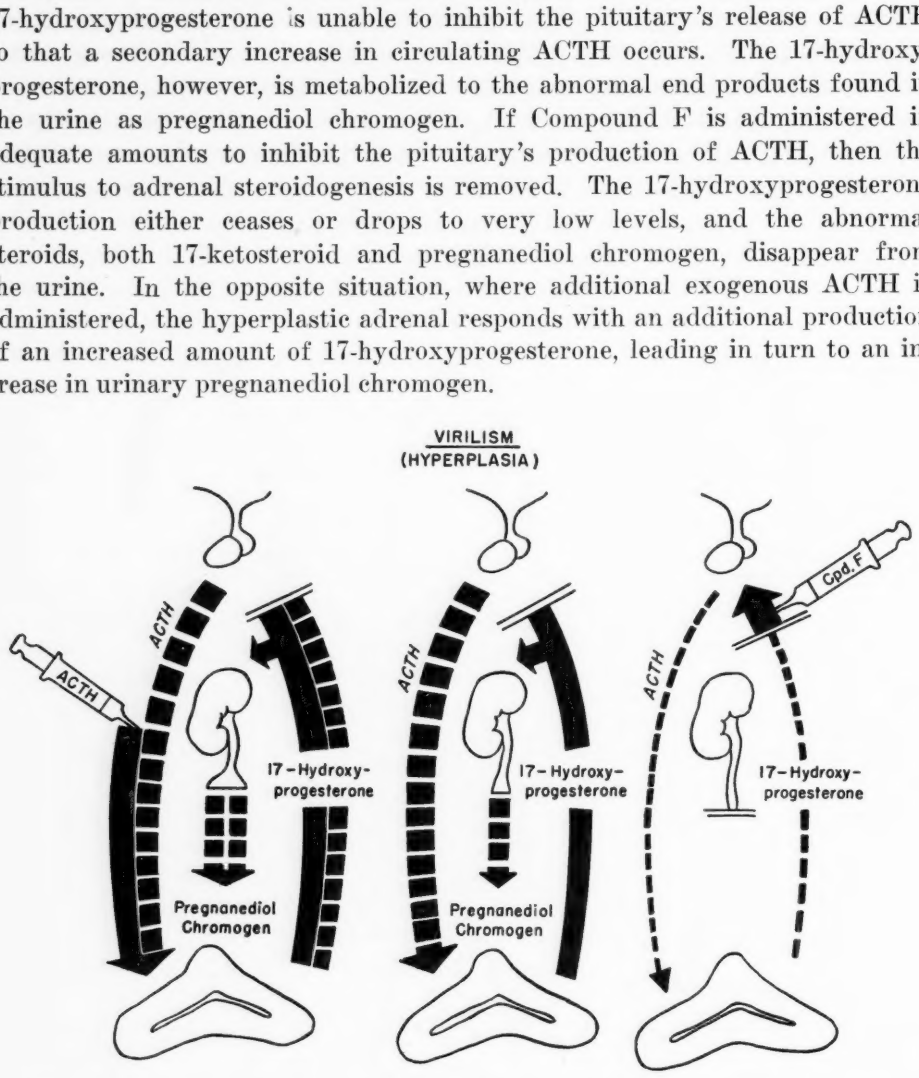


Fig. 5.—Adrenal pituitary axis on virilizing adrenal hyperplasia—as affected by exogenous ACTH or Compound F (hydrocortisone).

The preparation of Compound F employed contained 100 mg. in 500 ml. of 5 per cent dextrose in 1 per cent alcohol. In our earliest studies we employed a continuous injection apparatus. However, we found it simpler to utilize a disposable plastic intravenous set with a 25 gauge needle leading into a three-foot length of polyethylene tubing from which a 25 gauge cannula is inserted into the vein. The rate of flow can be controlled by adjusting the

height of the infusion bottle. We have found that one drop every 17 to 19 seconds will deliver approximately 250 ml. (50 mg. Compound F) in a 24-hour period.

Fig. 6 illustrates the effect of the intravenous administration of Compound F continuously over a 24-hour period in a total dose of 50 mg. in M. B., a 17-year-old white girl. Her chief complaints were those of acne, primary amenorrhea, hirsutism, and a failure of breast development. She had never menstruated in spite of several courses of estrogen therapy. Physically, she was a short, somewhat stocky girl, blood pressure 140/78, who showed marked hirsutism over face, arms, legs, and trunk. The muscular development was quite masculine. No breast tissue was palpable. Generalized acne was present and definite enlargement of the clitoris was seen upon pelvic examination. The ovaries could not be felt. Chest x-ray and excretory urograms were normal. The urinary 17-ketosteroid excretion was 39 mg. every 24 hours, and on cortisone therapy, 50 mg. intramuscularly daily, a drop to normal ketosteroid excretion was seen, associated with a clinical remission of the symptoms. The urinary excretions of 17-ketosteroid and pregnanediol chromogen during the intravenous administration of Compound F are shown in Fig. 6.

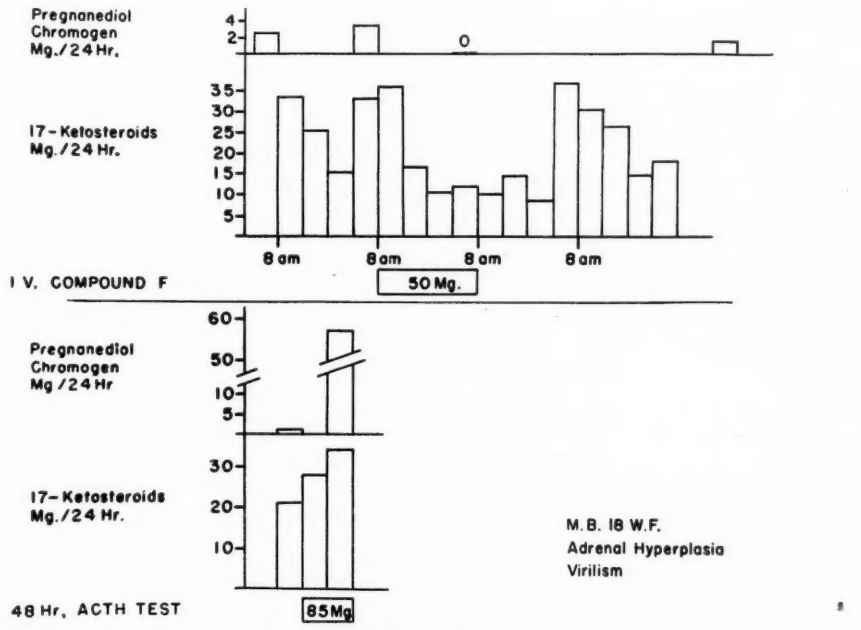


Fig. 6.—The influence of intravenous Compound F and intramuscular ACTH upon steroid excretion in virilizing adrenal hyperplasia.

It can be seen very readily that the administration of Compound F led to a substantial reduction in the rate of excretion of both of these steroids. The diurnal variation observed in the urinary 17-ketosteroid excretion is quite evident and is also to be noted in Fig. 7.

When ACTH was administered in our standard 48-hour ACTH test,[6] the opposite response was obtained. It is of interest that the increased pregnanediol chromogen excretion was much greater than was the increase in 17-ketosteroid excretion.

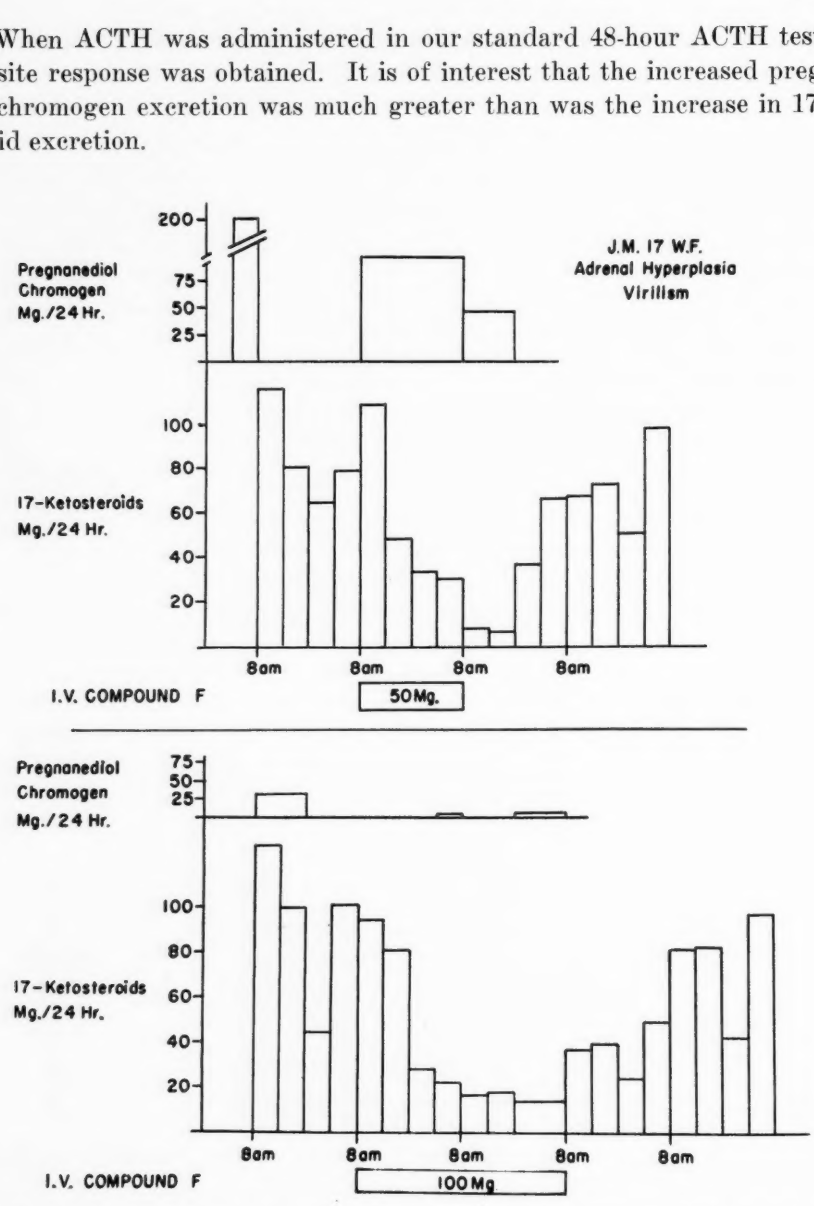


Fig. 7.—Comparison of effect of 24- and 48-hour infusions of intravenous Compound F upon steroid excretion in virilizing adrenal hyperplasia.

Fig. 7 shows this same type of test, as well as the result in a second patient with adrenal hyperplasia and virilism (J. M.). She was a 17-year-old girl whose complaints were primary amenorrhea, hirsutism since the age of 8 years, deepening of the voice since the age of 11 years. Physical examination revealed generalized body hirsutism, atrophic breasts, and an enlarged clitoris. No definite ovarian tissue could be palpated. Excretory urograms were normal, and the urinary excretion of 17-ketosteroid was 163.0 mg. every 24 hours.

Intramuscular cortisone acetate therapy resulted in a clinical as well as a laboratory remission. The effect of the intravenous administration of Compound F is shown in Fig. 7. The diurnal variation in 17-ketosteroid excretion is to be noted, as well as the very substantial drop in ketosteroid excretion seen immediately after the 24-hour infusion of 50 mg. of Compound F was discontinued. The decrease in pregnanediol chromogen excretion was also substantial.

In this same patient an attempt was made to extend the infusion of hydrocortisone over a 48-hour period, allowing 100 mg. to be infused: the results can be seen to approximate those of the 50 mg. infusion. Therefore, it can be seen that there is little substantial advantage to be gained by lengthening the infusion time as well as the dosage. In other words, the necessary information can be obtained from the 24-hour infusion test.

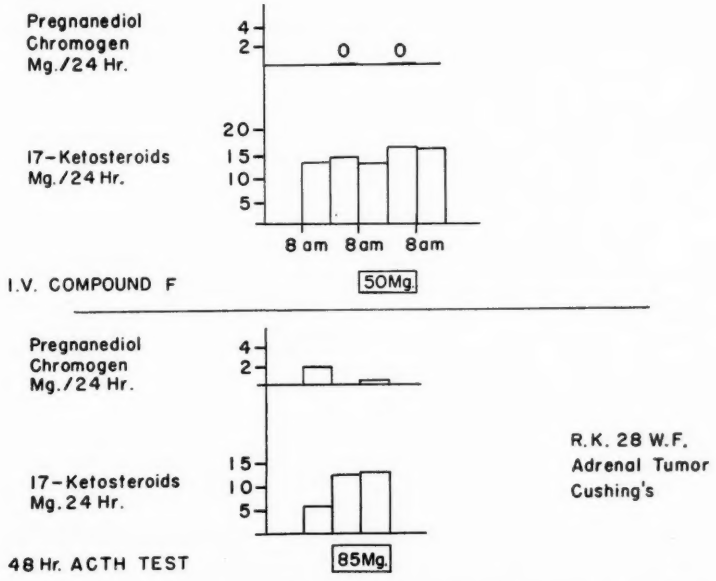


Fig. 8.—The influence of intravenous Compound F and intramuscular ACTH upon steroid excretion in a patient with Cushing's syndrome due to malignant adrenal tumor.

The data on a patient with Cushing's syndrome (R. K.) are shown in Fig. 8. She was a 28-year-old white woman who proved at operation to have a malignant adrenal tumor, accounting for the Cushing's syndrome. In this patient it can be seen that there was no variation induced in the 17-ketosteroid excretion by the administration of the hydrocortisone. The only change noted was following ACTH testing, which resulted in a slight decrease in pregnanediol chromogen excretion and a modest increase in 17-ketosteroid excretion. The increased 17-ketosteroid excretion was interpreted as resulting from the normal adrenal response of nontumor tissue.

An additional patient with adrenal hyperplasia and virilism has been studied with this same method of administration of hydrocortisone, and similar

results have been obtained. A virilized patient, who on surgical exploration was found to have Stein-Leventhal-type ovaries, showed no significant change in steroid excretions when the intravenous hydrocortisone test was performed.

DISCUSSION

The intramuscular administration of cortisone over a period of time has been shown to decrease the abnormal production of steroids in virilizing adrenal hyperplasia[7] and to have little or no effect on the steroid production from adrenal tumors.[8] This has resulted in the usage of intramuscular cortisone as a means of differentiating adrenal tumors from adrenal hyperplasia.[9]

The intravenous administration of hydrocortisone as described in this paper has been shown to have the same effect in these diseases as would be expected from the intramuscular administration of cortisone. Furthermore, these results can be obtained in a much shorter period of time and with considerably less expense and trouble to the patient. We, therefore, believe that the use of hydrocortisone administered by continuous 24-hour intravenous route is a practical clinical means of differentiating virilizing adrenal hyperplasia from other adrenal lesions and nonadrenal virilizing conditions.

We do not as yet have any information on the effect of the intravenous hydrocortisone upon an adrenal tumor producing virilism. However, we would expect that the infusion would result in no change in steroid excretion because in one such patient of ours, intramuscular cortisone therapy failed to influence the 17-ketosteroid excretion. We have seen one patient recently who had proved hyperplasia associated with Cushing's syndrome and in whom the intravenous hydrocortisone produced no change in steroid excretion.

SUMMARY

Current theories as to the etiology of adrenal hyperplasia with virilism have been discussed. The adrenal pituitary relationships in this disease, in adrenal tumors with virilism, and in hyperadrenal states resulting in Cushing's syndrome have been illustrated.

The intravenous administration of hydrocortisone over a 24-hour period has been shown to have a marked inhibitory effect on the excretion of the abnormal steroids found in adrenal hyperplasia with virilism and to have little or no effect upon adrenal tumors. This type of test has been proposed as a rapid means of differentiating virilizing adrenal hyperplasia from other adrenal lesions and nonadrenal virilizing conditions. It is believed that this is a much more rapid, and, therefore, more convenient test than is the intramuscular administration of cortisone acetate in differentiating these hyperadrenal states.

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OBSERVATIONS ON "ISOMETRIC" CONTRACTION OF THE ATRIA IN THE HUMAN SUBJECT

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WHEN the right atrium contracts, blood may be expelled through the tricuspid valve, the venae cavae, the coronary sinus, or any other available communication such as an atrial septal defect or an anomalous pulmonary vein. Because the greatest quantity normally leaves via the tricuspid valve, considerable alteration in the hemodynamics may be expected during atrial contraction when the tricuspid orifice is closed. Right atrial pressure pulses recorded during cardiac catheterization of three patients with widely different cardiac lesions provided an unusual opportunity for analyzing such altered atrial dynamics.

Large right atrial "a" waves were observed in all these cases. These were shown to be due, in part, to resistance to backflow of blood through the venae cavae, caused by increased momentum of venous return during the latter part of ventricular systole. The use of the term "isometric" contraction of an atrium is discussed in the light of the findings. In addition, further evidence is presented for the reflection of left atrial pressures in pulmonary wedge pressure pulse contours under some circumstances.

METHOD

The pressure pulses were recorded simultaneously with the electrocardiogram during cardiac catheterization by means of Sanborn capacitance manometers on a four channel Polyviso recorder. The zero-level reference point for the atrial pressure was taken as 5 cm. dorsad to the angle of Louis in each case. In one case right atrial and right ventricular pulses were simultaneously recorded; in the other two, their time relationship was obtained by plotting them against each other at comparable heart rates using the simultaneously recorded electrocardiogram as the time reference.

Material.—Case 1 was a 12-year-old girl with severe pulmonic valvular stenosis, an intact ventricular septum, and a patent foramen ovale, who developed runs of atrioventricular nodal rhythm while the catheter tip was in the right atrium. Case 2 was a man, aged 25 years, who had complete heart block (probably congenital) with a variety of pacemakers arising below the bifurcation of the common A-V bundle. Case 3 was a man, aged 52 years, with mitral stenosis and a persistent auricular tachycardia with different grades of A-V block, the ratios varying from 1:1 to 3:1.

Observations.—

Case 1.—The right atrial pressure pulses during sinus and nodal rhythm are shown in Fig. 1, *A* and *B*. An augmented atrial contraction wave is present during sinus rhythm (rate, 125 per minute) in the right atrial pressure pulse (Fig. 1, *A*). This "a" wave of about 5 mm. Hg begins its rise coincident with the summit of the P wave, and reaches its peak 0.08 second later at the same time as the right ventricle reaches its diastolic level (Fig. 2, *A*).

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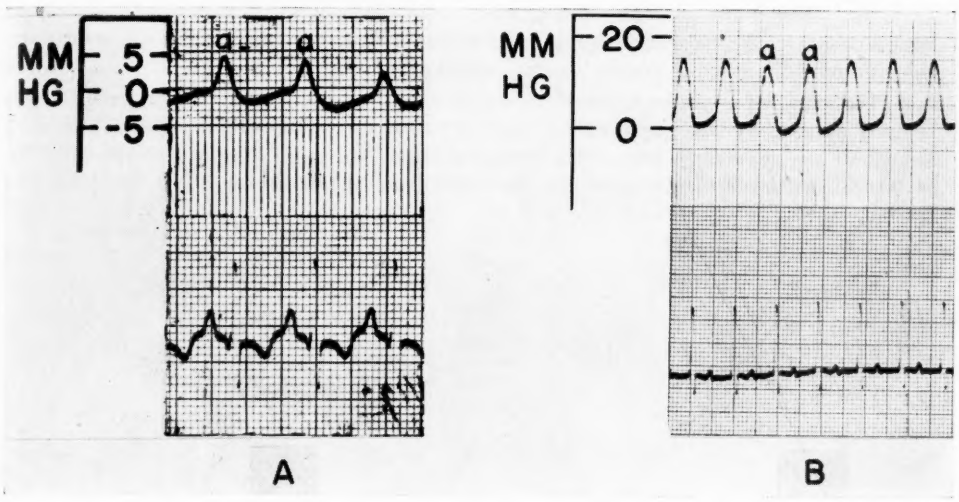

Fig. 1.—*A* and *B* are both from Case 1 and are reproduced to different scales. In each the right atrial pressure pulse is above (with calibration at the left) and Lead II of the electrocardiogram below; the "a" wave is labeled. In *A* there is sinus rhythm (rate 125); in *B*, nodal rhythm (rate 136). (Discussed in text.)

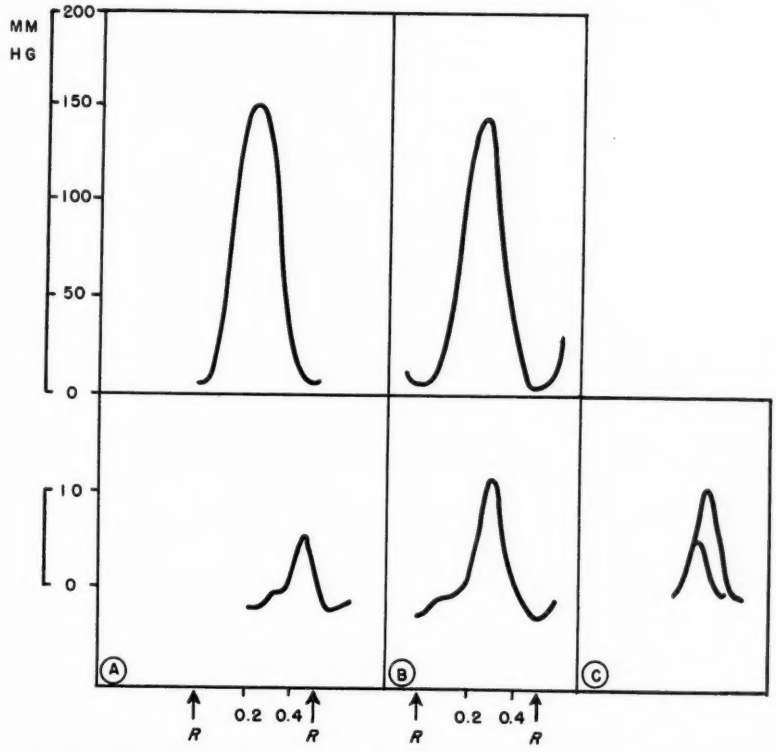

Fig. 2.—The time relationship between atrial and ventricular systole shown diagrammatically from reconstruction of original records of right atrial and right ventricular pressure pulses. *A* shows the relationship during sinus rhythm; *B* during nodal rhythm. In each the ventricular curve is above and the atrial curve is below. Ordinates in millimeters of mercury on left, R wave peak indicated below by ↑, time from first R wave given in seconds. In *C* the atrial pressure pulses of *A* and *B* are superimposed. (Discussed in text.)

Thus, due to the rapid heart action, the right ventricular pressure does not reach its diastolic level until after the atrial systole is partially over, and the tricuspid valve apparently does not open until the peak of the "a" wave is reached.

The "a" wave of the right atrial pressure pulse during nodal rhythm (rate, 136 per minute) (Fig. 1, *B*) is larger (11 to 12 mm. Hg) than during sinus rhythm. It begins at the summit of the apparently retrograde P wave in Lead II. Fig. 2, *B* shows that atrial and ventricular maximal systolic pressures are reached almost simultaneously, and so are their diastolic levels.

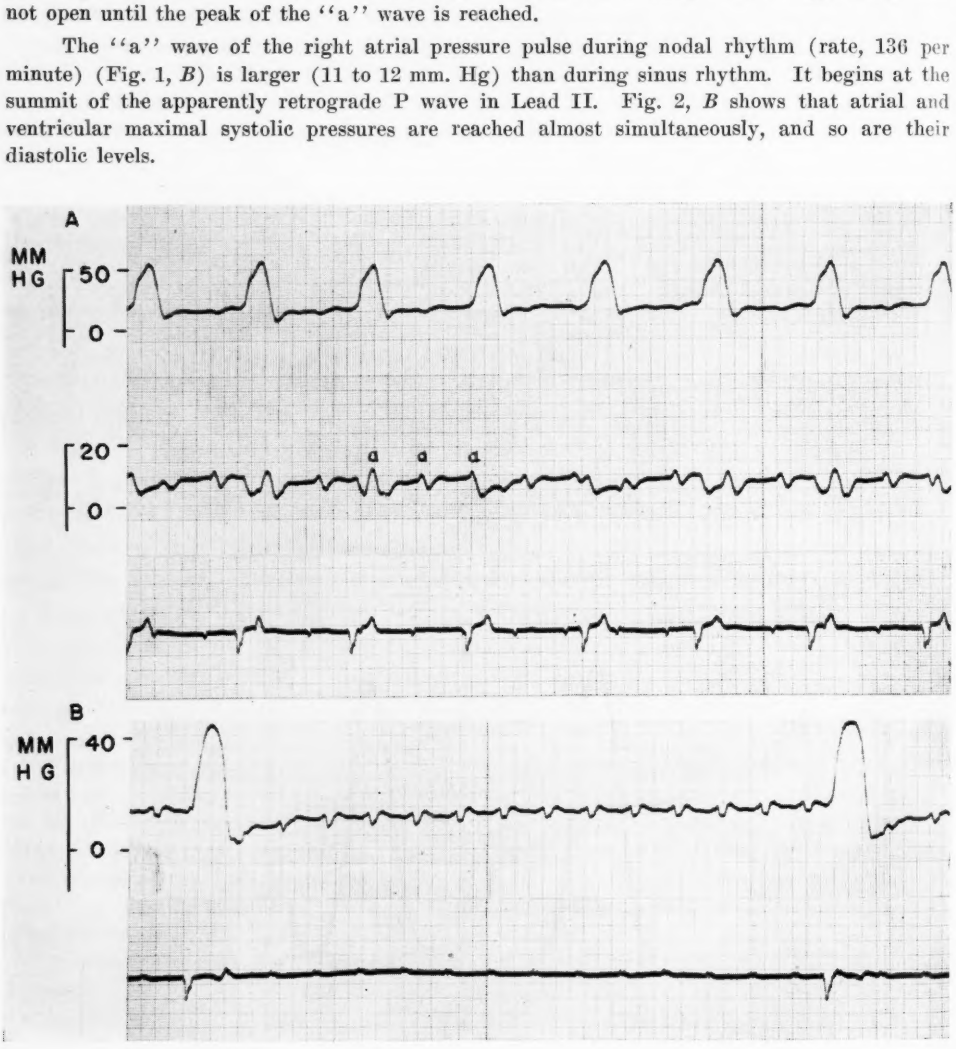

Fig. 3.—Records taken in Case 2. Before *B* and after *A* was a short period of ventricular paroxysmal tachycardia, which accounts for the change in P and QRS contour between the two records.

In *A* from above downward, are shown right ventricular pressure pulse, right atrial pressure pulse (with "a" waves labeled), and Lead II of the electrocardiogram. In *B*, the records are the same but no right atrial pressure pulse is shown. In both records the calibration is on the left; the heavy time lines are 0.2 second apart. (Discussed in text.)

CASE 2.—The auricular rate was recorded at 84 or 94 beats per minute (Fig. 3), but was constant through any ventricular cycle, neither speeding up during long ventricular pauses, nor slowing following a ventricular contraction. The ventricular rate varied between 6 and 24 beats per minute. With each P wave of the electrocardiogram there were diphasic waves in the right atrial pressure pulse (Fig. 3, *A*); each consisted of a 2 mm. Hg positive "a" wave lasting 0.08 second, followed by a 3 mm. Hg "diastolic dip" which lasted 0.16 second. These waves were identical with one another as long as they occurred in ventricular diastole. Right atrial systolic waves were also seen in the right ventricular pulse during diastole when recording was done with sufficient sensitivity (Fig. 3, *B*).

In order to assay the true character of the right atrial pressure pulse during right ventricular activity, it was first necessary to isolate the right atrial pressure curve during this period from the "a" waves and auricular "diastolic dips" which constantly dominated it. It was possible to do this by superimposing several ventricular portions of the right atrial curve of Fig. 3, *A*, each having "a" waves at different times in the ventricular cycle, and by using the part of each curve just before the "a" wave, and thus to arrive at a sound impression of what the contour would have been during the whole period in the absence of auricular activity (Fig. 4, *A* and *B*). With this as a base line, the auricular systolic pressures could be derived by algebraically subtracting the basic ventricular effect from the actual atrial curve recorded (Fig. 4, *C*). By this means, large "a" waves, ranging up to 10 mm. Hg, each followed by a "diastolic dip" were demonstrated in the right atrial pulse during ventricular contraction (Fig. 4, *B* and *C*). During the periods of isometric contraction and reduced ejection of the right ventricle the "a" waves were only slightly augmented, although the tricuspid valve was closed, but during the latter part of ventricular systole they show definite augmentation.

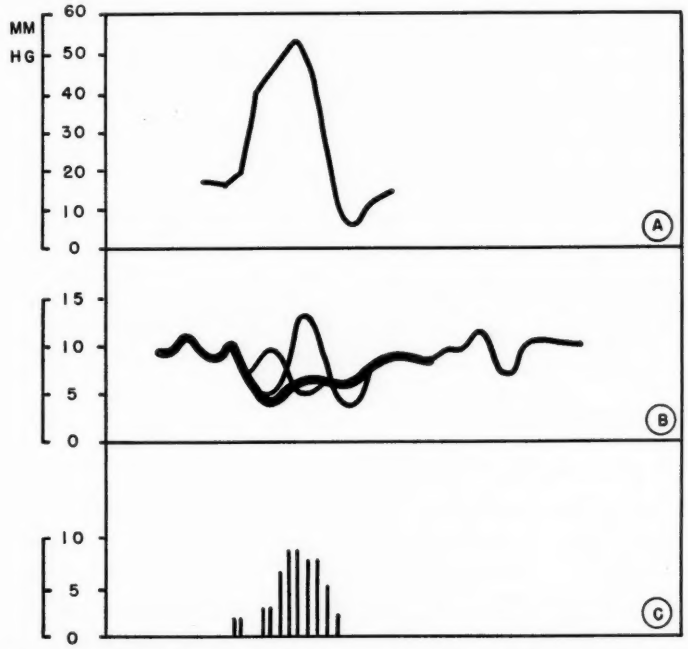

Fig. 4.—Reconstructions based on Fig. 3, *A*. Calibrations in millimeters of mercury on the left. *A*, *B*, and *C* are constructed so as to be synchronous in time. *A* is the right ventricular pressure pulse. *B* shows the right atrial pressure pulse (heavy line) as it would look were the effects of atrial activity eliminated during ventricular activity. The several lighter lines superimposed show the "a" waves and the atrial diastolic dips of several atrial beats. *C* shows the variation in peak of the "a" waves through ventricular systole. Each bar represents several determinations—which were remarkably constant. (Discussed in text.)

CASE 3.—Fig. 5, *A* illustrates the auricular tachycardia, which is not well shown in the electrocardiogram recorded by the Poly-viso. In Fig. 5, *B* the auricular rate is 167 per minute with 2:1 A-V conduction. Two types of "a" waves are present and they occur alternately. The large "a" waves have an amplitude of 8 mm. Hg on the average, compared to little more than 1 mm. Hg for the smaller ones. Synchronization with the right ventricular pulse curve, as in Case 1 discussed above, showed that the large "a" waves begin shortly after the onset of ventricular systole, and that the anacrotus of both contractions are of about the same duration. The small right atrial "a" waves begin after the right ventricular pressure pulse returns to its diastolic level and therefore occur following the opening of the tricuspid valve.

Under such circumstances, two types of "a" waves might be expected to occur also in the left atrial pulse tracing, but no left atrial pressure pulse tracing was recorded. In Fig. 5, *C*, a pulmonary wedge pressure pulse is shown, and a large pressure wave of 15 to 18 mm. Hg amplitude is seen to occur at a time corresponding to the nonconducted P wave. The alternate "a" waves are not discernible.

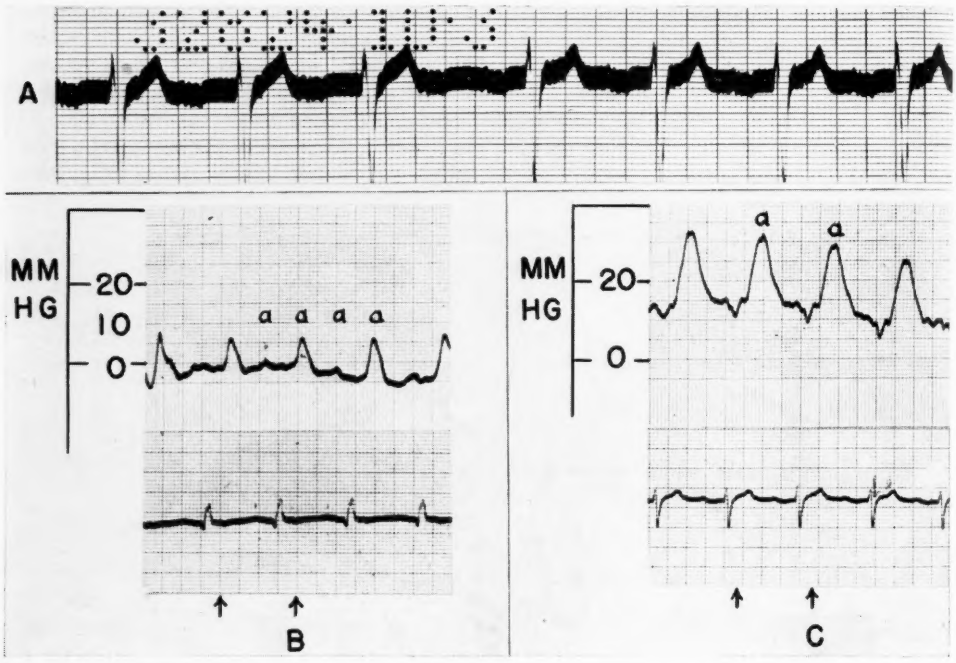

Fig. 5.—*A*, *B*, and *C* are all from Case 3. *A* shows Lead III of the electrocardiogram during auricular tachycardia with varying degrees of A-V block. The ectopic P waves occurring at a constant rate of 150 beats per minute (as can be demonstrated by calipers) are clearly seen, especially in the long pause. *B* and *C* are Poly-viso records. In *B* right atrial pressure pulse is above and Lead II below. In *C* pulmonary wedge pressure pulse is above and Lead I below. The calibration of the pressure pulses are on the left, time is 0.2 second between heavy lines. The "a" waves are labeled. (Discussed in text.)

DISCUSSION

In the present study, pressure tracings of atrial contractions against closed A-V valves were obtained, and these showed, for the most part, markedly augmented "a" waves. It is important to determine the extent to which such atrial contractions approach the isometric, particularly since the question of reflex closure of the caval orifices during atrial systole has been raised.[1]

The term isometric contraction applied strictly to any chamber of the heart means that none of the energy expenditure of contraction results in displacement of the contained blood. Neglecting friction, all of the energy in an isometric contraction is expressed as a pressure rise within the chamber. Therefore, isometric contraction of an atrium should manifest itself as an augmented "a" wave in its pressure pulse.

The factors governing the atrial pressure reached during atrial systole under usual circumstances are well known.[2] Theoretically, during an isometric

atrial contraction the atrial pressure depends only on the energy expended by the atrium, which in turn depends upon the amount of blood present in the atrium at the onset of atrial contraction.[3] Further, the volume and pressure of the relaxed atrium do not bear a linear relationship to each other.[4]

Augmented "a" waves may occur during ventricular systole or diastole. Such augmented "a" waves are presystolic in tricuspid stenosis or in severe right ventricular hypertension with intact ventricular septum and are systolic during most types of nodal rhythm and in partial or complete A-V dissociation. This discussion will be confined to those augmented "a" waves occurring during ventricular systole.

Case 2 (Figs. 3 and 4) shows that when the atrium contracts very early in ventricular systole the atrial systolic pressure is augmented by no more than 1 mm. Hg over that occurring in ventricular diastole even though the summit of "a" wave occurs after the tricuspid valve closes. Thus, right atrial contraction against a closed tricuspid valve per se does not mean isometric contraction, presumably because reflux through the venae cavae can still occur even though venous return must first be stopped and reversed. Only after ventricular ejection is well under way and pronounced, and leads to an increased venous return to the right[5] and left[6-8] heart, does the "a" wave become markedly augmented.

The augmentation of the "a" waves found in the right atrial pulse in Case 2 from the time of maximal ventricular ejection until the tricuspid valve opens is due to several factors, namely: (1) the closed tricuspid valve; (2) the greater force of contraction dependent upon the greater amount of blood in the right atrium at this time despite its lower precontraction pressure; (3) the greatly increased momentum of the blood returning to the heart. This increased momentum which has to be overcome before reflux can occur acts as a functional check valve, and the impact of the atrial contraction on this functional impediment results in the higher atrial contraction pressure. This impact wave would be transmitted peripherally through the venae cavae.

One would expect a negligible reflux, neglecting the coronary sinus, from the right atrium under these circumstances. If so, such an atrial contraction is isometric. However, considerable reflux into the venae cavae and the coronary sinus has been demonstrated in one case by angiocardiography when the right atrium contracted under similar circumstances in a case of complete A-V block.[1] No mention is made in the foregoing report as to whether such a reflux occurred during all of the atrial systoles or only in those when atrial and ventricular systoles occurred simultaneously. It is none the less clear from Case 2, whether or not the augmented "a" wave is fully isometric, that increased venous return played a large part in its development with the tricuspid valve closure in a supporting role (Fig. 4). It is also clear that had caval closure reflexes been operating, any "a" wave augmentation should occur from the instant that the tricuspid valves closed rather than only at some time later in ventricular systole. This is because such reflexes, if dynamically important, should manifest themselves by annulling the effects of increased venous return.

Case 1 (Fig 1, *A* and *B*) shows that the augmented "a" waves of the trilogy of Fallot may become much larger during nodal rhythm at a rate approximating the previous sinus rhythm. Since Case 2 has shown that closure of the tricuspid valve alone has little effect upon right atrial systolic pressure, the large difference in size between the two types of "a" wave would not be expected. This is because the effects of increased venous return would be annulled by caval reflexes were these reflexes present. The patent foramen ovale found in this case may not be a significant pressure-reducing factor because the left atrium is undoubtedly undergoing similar alteration in its systolic pressures. Incidentally, the volume of the atrium is smaller just before its contraction during nodal rhythm than during sinus rhythm because atrial contraction of nodal rhythm commences earlier in the period of greatest venous return. Therefore, at this rapid rate, the actual force of the high-pressure contraction is probably less than that of the low-pressure one, unless afterloading of the atrium can affect its force of contraction.

There is one other cause of large "a" waves illustrated by Case 1. The heart rate is fast and measurements show that the earlier part of the sinus atrial systole occurs before the A-V valve opens. Since, in addition, it is at a time of increased venous return, the early part of the contraction is isometric and the ascending limb of the curve has the same slope as the nodal "a" wave (Fig. 2, *C*). However, the pressure of the "a" wave of nodal rhythm is not reached because, as closely as can be measured, the "a" wave, as it were, collapses as the tricuspid valve opens. Fig. 2, *A* and *B*, shows that the peaks of both types of "a" wave occur late in ventricular systole, but the sinus "a" wave is relatively later and is similar to the other (Fig. 2, *C*) except that its upstroke is cut off by the opening of the tricuspid valve. This illustrates well the supporting role of the closed tricuspid valve in the development of these large "a" waves.

In Case 3, the pressure of right atrial systole against the closed tricuspid valve is eight times the pressure under usual circumstances (Fig. 5, *B*), and again every second atrial contraction is isometric, or nearly so. There is no basis for the comparison of the atrial volumes before the high- and low-pressure atrial contractions, although they probably differ little. It is unlikely that any slight changes in energy expenditure of contraction based on atrial end-diastolic fiber length alone can play a part in such great changes in pressure. Closed A-V valves and increased venous return during atrial systole constitute augmented afterloading of the atrium. Here, the atrial musculature has a greater afterloading during every second contraction. Afterloading of ventricular muscle may to some extent affect the energy expenditure of the ventricular contraction independently of initial fiber length.[9-11] Such a phenomenon is possibly acting on atrial muscle here, as it may during nodal rhythm, or during a developing tricuspid stenosis.

In accounting for the unusual form of the pulmonary wedge pressure of Case 3 shown in Fig. 5, *C*, it is reasonable to assume that due to the patient's arrhythmia much the same events are going on in the left atrium as just described in the right atrium. The best explanation of the large "a" wave lies

in its representing the impact of left atrial contraction against a closed mitral valve, and against the increased pulmonary venous return associated with ventricular systole. Despite the fact that this patient has mitral stenosis, the segment of the circulation between the pulmonary venous capillaries and the mitral valve has less volume than the corresponding segment which includes the right atrium and the venae cavae. Further, the elevated over-all pressure level between "a" waves indicates distention of this segment. These latter two facts contribute further to the large impact wave in the pulmonary wedge curve, which thus is a manifestation of "isometric" contraction of the left atrium.

The circumstances of this observation provide further indication that the pulmonary wedge pressure can be a reflection of left atrial pressure under some circumstances. There is no reason to consider any significant degree of mitral regurgitation as being responsible for the contour of this pressure pulse, because there was no clinical suspicion whatever of mitral regurgitation, nor are the time relationships of these large systolic waves to the pulmonary arterial pressure curves those commonly seen in mitral regurgitation.

The exaggerated pulsations seen in the jugular veins in instances of nodal rhythm do not favor the idea of caval closure reflexes occurring under these circumstances. As Lind and his group[1] point out, quoting Wiggers,[3] experimental evidence favors the impression that the mouths of the venae cavae are open throughout the cardiac cycle. Although the presence of a catheter may well produce artifact, it seems that were caval closure reflexes associated with augmented "a" waves, it should be possible to demonstrate some gradient in the height of the atrial systolic wave across the mouth of the inferior or the superior vena cava. This has not been the case so far in our limited experience. Under normal circumstances, the angiocardiographic evidence of dye being held up at the mouth of the superior vena cava during atrial systole and flooding the atrial cavity immediately thereafter can be as readily attributed to interruption of filling due to atrial systole followed by the period of increased venous return, as to the supposed caval closure reflexes. In the present study, caval closure reflexes could also be ruled out as an important dynamic factor in certain pathologic instances.

SUMMARY

Right atrial pressure pulses were obtained together with additional physiologic data, by cardiac catheterization of three patients who shared, in common, the occurrence of virtually simultaneous contraction of the atria and ventricles either as an intermittent phenomenon or an event present during short periods.

Augmented "a" waves were observed in the atrial pressure pulse under these circumstances, and it was shown that increased venous return played a part in their development. Under some circumstances augmented "a" waves represent virtually isometric contraction of the atrium. It is unlikely that augmented pressure of atrial contraction necessarily means increased force of contraction.

In one of our cases, the expected pressure variations of the "a" wave of the left atrial pressure pulse were reflected in the pulmonary wedge pressure curve.

I am indebted to the members of the catheterization unit of the Department for assistance in obtaining these data, to the physicians of these patients for permission to report the cases, and to Drs. L. N. Katz, A. Pick, and E. Silber for advice in preparing this report.

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HORMONAL FACTORS IN THE PRODUCTION OF EXPERIMENTAL RENAL AND CARDIOVASCULAR DISEASE

(Synergism Between Somatotrophic Hormone and Methylandrostenediol)

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EARLIER experiments have shown that, in rats sensitized by unilateral nephrectomy and a high-sodium diet, electrophoretically homogeneous preparations of STH ("somatotrophic hormone" or "growth hormone") caused nephrosclerosis, polyuria, myocarditis, and hypertension. The sensitizing procedure alone, or STH given to nonsensitized rats, proved virtually ineffective in these respects. The syndrome caused by this hypophysial hormone very closely resembles that produced (under similar conditions of sensitization) by mineralo-corticoids, such as desoxycorticosterone acetate (DCA). However, one striking difference between the effects of STH and DCA is that the former stimulates somatic growth, but causes little or no periarteritis nodosa, while the latter produces marked periarteritic changes, but no excess growth. If rats are simultaneously treated with moderate doses of STH and DCA, the resulting syndrome of nephrosclerosis, polyuria, myocarditis, and periarteritis nodosa is of quite extraordinary severity.[16] It was, therefore, concluded that the actions of such steroids as DCA are enhanced by the "mineralo-corticoid conditioning effect" of STH.[1, 2]

A great many investigations in this and several other laboratories had shown that pituitary preparations devoid of STH and steroids possessing no mineralo-corticoid potency fail to elicit the previously mentioned pathologic changes.[3-7] Hence, it seemed reasonable to assume that, at least in the rat, the production of these changes is rather specifically related to mineralo-corticoid activity, although subject to such conditioning factors as sodium and STH.

More recently, it has been observed, however, that a synthetic androgen, methylandrostenediol (MAD), can also produce essentially similar cardiovascular and renal changes.[8] This compound is devoid of corticoid activity, as judged by life-maintenance tests, in adrenalectomized rats.[9] In the light of these observations, it was quite justified to doubt the validity of the concept which ascribes the production of these changes more specifically to mineralo-corticoids.[10]

This seemingly incongruous finding has now been clarified to a large extent. It could be shown that although MAD produces nephrosclerosis, periarteritis nodosa, myocarditis, hypertension, and polyuria of the same type (and under the same conditions of sensitization) as DCA, unlike the latter, it does so only in the presence of the adrenals. Presumably this synthetic steroid acts by inter-

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fering with the normal secretory activity of the adrenal, inducing the latter to produce an excess of mineralo-corticoid (DCA-like) hormones. Thus the observed toxic actions actually would not be those of MAD, but those of endogenous DCA-like steroids. This view was further strengthened by the finding of rather characteristic histologic changes in the adrenal cortices of MAD-treated animals, namely, the accumulation of numerous, intensely PAS-positive, cytoplasmic granules.[11-13]

We should like to report now upon a series of experiments designed to analyze the mechanism through which MAD produces its toxic effects upon the heart and the cardiovascular system. In particular, we were interested to see whether STH augments the toxicity of MAD in the same manner as it enhances that of DCA, and whether the singular histologic changes, produced by MAD in the adrenals, can be altered by simultaneous STH treatment.

MATERIALS AND TECHNIQUES

Eighty female Sprague-Dawley albino rats were subdivided into 8 equal groups (each with an average initial body weight of 110 grams), as outlined in Table I. In Groups V to VIII the right kidney was removed at the beginning of the experiment.

Two milligrams of MAD (17 α-methyl-Δ^5-androstene-3 [β], 17 [β]-diol) microcrystals were administered daily, subcutaneously, in 0.3 ml. of water, Tween 80 being used as a suspending agent.

STH* was administered twice a day subcutaneously, dissolved in saline solution. The daily dose was 1 mg. per animal from the beginning of the experiment and was increased to 2 mg. on the twenty-first day and to 4 mg. on the twenty-eighth day.

The animals of the first four groups received tap water ad libitum to drink, whereas those of the last four groups received 1 per cent NaCl as drinking fluid. All animals were given Purina Fox Chow ad libitum.

Blood pressure measurements were made at frequent intervals by the tail-cuff method of Friedman and Freed.[14]

All animals were killed after 52 days of treatment and their organs were fixed in Susa for weighing and histologic study. Sections were stained with hematoxylin-eosin as well as with the PAS procedure.[15]

The statistical evaluation of our data was made according to Snedecor.[17]

RESULTS

In the course of the experiment, it soon became evident that the blood pressure and the *fluid intake* (as well as the diuresis) rose significantly above normal only in sensitized (unilateral nephrectomy and saline) rats treated with MAD and/or STH. In both these respects combined treatment with MAD and STH was most effective. In general, the blood pressure effect slightly preceded that of the fluid turnover (Fig. 1).

The *somatic-weight* increase, on the other hand, was essentially the same in sensitized and nonsensitized animals. However, among the latter, the growth rate was somewhat impeded in the group receiving both STH and MAD, presumably because of the systemic toxic effects of this combined treatment.

*Armour Lot R-285-183.

In Table I the mean organ weights (with their standard errors) are listed (both in absolute terms and in milligrams per 100 grams of body weight) together with the most striking pathologic changes. The severity of the latter is expressed in a scale ranging from "0" to "+++."

The weight of the one remaining *kidney* in the unilaterally nephrectomized groups was almost the same as that of the two kidneys in intact animals receiving the same treatment. Both, when expressed in absolute terms and as milligrams per 100 grams of body weight, MAD alone, or STH alone, caused some renal enlargement above the controls, but the effect of simultaneous treatment with MAD and STH was much more pronounced.

Nephrosclerotic changes were not observed in any of the intact animals (Groups I to IV), nor in the sensitized controls (Group V). They were of moderate intensity after MAD (Group VI) or STH (Group VII) alone, and of very severe nature in the rats receiving both hormones (Group VIII) after unilateral nephrectomy and a high NaCl intake (Fig. 2).

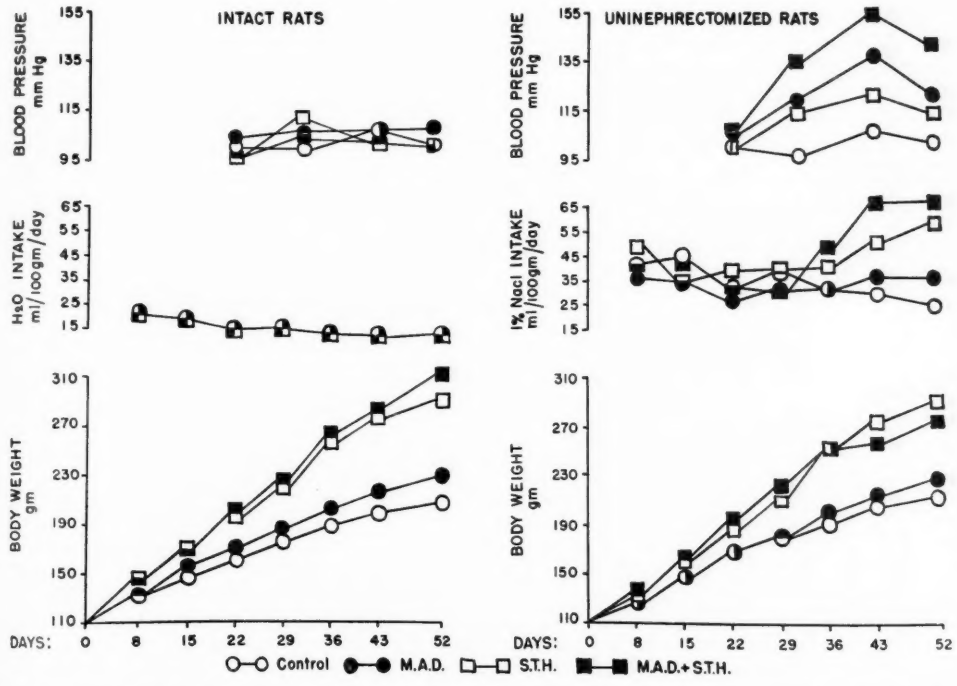

Fig. 1.—Changes in body weight, fluid intake, and blood pressure in rats treated with STH and/or MAD.

The *heart* weight, as well as the development of myocarditis, ran closely parallel to the previously mentioned renal changes (Fig. 3).

The *adrenals* of the MAD-treated, intact animals did not significantly differ in weight from the normals. However, STH caused a slight, but definite, increase in adrenal weight in the intact and particularly in the sensitized animals. This is not unexpected, because STH is known to cause adrenocortical hyper-

TABLE I. CHANGES IN ORGAN WEIGHTS AND PATHOLOGIC EVALUATION

GROUP		I	II	III
TREATMENT		NONE	MAD	STH
KIDNEY	in mg.	1,451 ± 29	1,969 ± 131	1,870 ± 54
	in mg. × 100 Gm.	694 ± 13	861 ± 21	652 ± 15
Nephrosclerosis		0	0	0
HEART	in mg.	710 ± 13.7	781 ± 14	946 ± 37
	in mg. × 100 Gm.	340 ± 3.5	342 ± 8	330 ± 16
Myocarditis		0	0	0
ADRENALS	in mg.	50 ± 1.16	50 ± 2.3	60 ± 3
	in mg. × 100 Gm.	24 ± 0.74	22 ± 0.24	21 ± 1.2
"PAS+" Granules		0	trace	0
SPLEEN	in mg.	493 ± 25	544 ± 25	689 ± 42
	in mg. × 100 Gm.	237 ± 15	238 ± 8.5	239 ± 11.6
THYMUS	in mg.	256 ± 17	121 ± 13.6	411 ± 40
	in mg. × 100 Gm.	123 ± 9	53 ± 5.5	143 ± 15.5
HYPOPHYSIS	in mg.	10.2 ± 0.63	7.1 ± 0.27	8.7 ± 0.33
	in mg. × 100 Gm.	4.9 ± 0.07	3.1 ± 0.01	3.0 ± 0.16
PREPUTIAL GLANDS	in mg.	107 ± 14	309 ± 23	177 ± 11
	in mg. × 100 Gm.	51 ± 5	134 ± 8	62 ± 5

*Groups V to VIII were sensitized to the mineralo-corticoid type of effect by unilateral

trophy in animals which are not hypophysectomized, presumably through the stimulation of an ACTH discharge.[4] It is noteworthy, however, that MAD actually diminished the adrenal weight in the sensitized animals. This diminution was significant not only when compared with the intact-untreated (Groups I and IV, $p < 0.01$) or intact-MAD-treated (Groups II and VI, $p < 0.01$), but probably even when compared with the sensitized-not-hormone-treated rats (Groups V and VI, $p = 0.03$). In the animals receiving MAD together with STH, this cortical atrophy was counteracted by the hypophysial hormone.

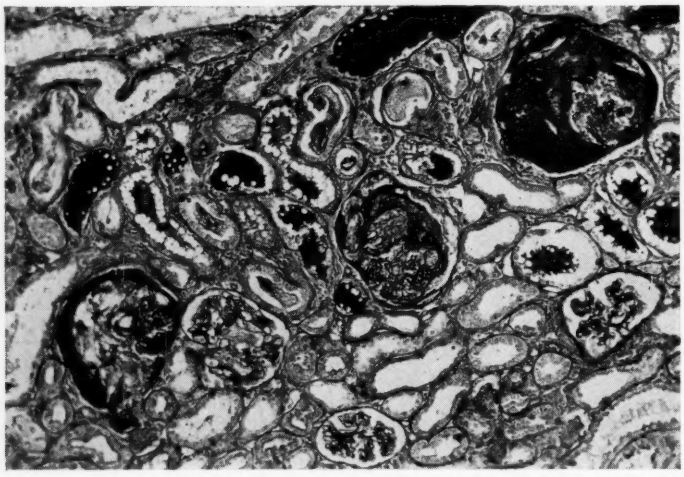

Fig. 2.—Kidney of an STH-plus-MAD-treated sensitized rat (Group VIII). Pronounced glomerular damage with PAS-positive material in the capsular spaces and in the convoluted tubules. (PAS stain ×100, reduced ⅙.)

Histologically, the most striking adrenal change was the development of those PAS-positive granules in the cortex, which are rather characteristic of MAD-treated animals, as outlined in our introductory remarks. It will be noted that such granules occurred only in MAD-treated animals and their develop-

OF LESIONS IN RATS TREATED WITH MAD AND/OR STH

IV	V	VI	VII	VIII
MAD + STH	NONE*	MAD*	STH*	MAD + STH*
2,392 ± 70	1,290 ± 42	1,747 ± 41	1,810 ± 392	2,619 ± 519
760 ± 14	613 ± 17	796 ± 22	631 ± 23	1,031 ± 93
0	0	17%	28%	74%
953 ± 31	697 ± 22	805 ± 16	955 ± 65	1,063 ± 18
307 ± 4.4	331 ± 8	367 ± 11	332 ± 12	418 ± 23
0	0	5%	12%	37%
52 ± 1.56	47 ± 1.8	41 ± 1.8	70 ± 2.3	67 ± 3.3
17 ± 0.65	23 ± 0.86	19 ± 0.99	25 ± 0.74	27 ± 2.4
+	0	+	0	+++
748 ± 39	552 ± 15.6	627 ± 20	727 ± 53	861 ± 134
241 ± 11.6	262 ± 6	287 ± 14	253 ± 7.4	339 ± 59
129 ± 13.5	322 ± 13	115 ± 12	472 ± 37	73 ± 19
42 ± 3.7	154 ± 7.7	52 ± 4.7	165 ± 9.5	24 ± 6.5
6.0 ± 0.23	8.6 ± 0.25	6.3 ± 0.37	8.3 ± 0.77	4.6 ± 0.28
2.0 ± 0.28	4.1 ± 0.13	2.8 ± 0.2	3.0 ± 0.33	1.8 ± 0.17
431 ± 27	105 ± 6	236 ± 27	147 ± 12	360 ± 54
138 ± 7	50 ± 3.4	148 ± 12	52 ± 3.6	141 ± 15

nephrectomy and a high NaCl intake.

ment was enhanced, both by the sensitizing procedure (nephrectomy with NaCl) and by simultaneous administration of STH. Consequently, the greatest development of PAS-positive granules was seen in the sensitized rats given MAD plus STH. It may be added that, in the groups treated with MAD, there were also always two other rather striking changes, namely: (1) the cell membranes of the cortical cells were particularly distinct (somewhat like those of plant cells) and highly tingible with the PAS procedure; (2) the fibrous tissue of the

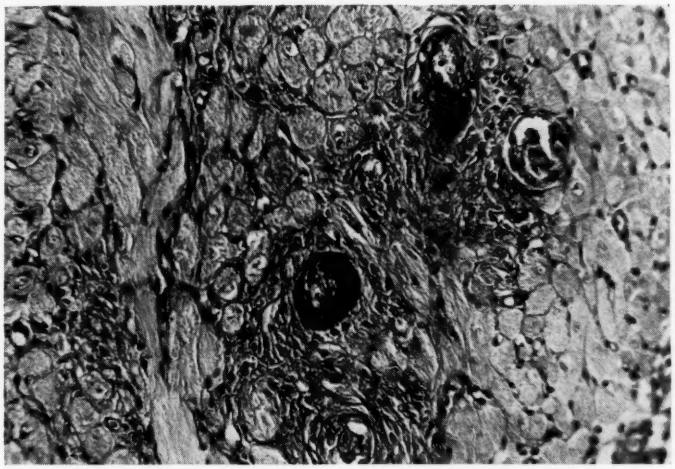

Fig. 3.—Heart of an STH-plus-MAD-treated sensitized rat (Group VIII). Cardiac periarteritis nodosa with accumulation of PAS-positive material in the vessel walls. (PAS stain ×250, reduced ⅙.)

adrenal capsule was extremely developed, highly tingible with PAS, and intermixed with comparatively undeveloped glomerulosa cells. The intensity of these changes showed a certain parallelism with the development of PAS-positive granules, but the latter lend themselves better to quantitative evaluation.

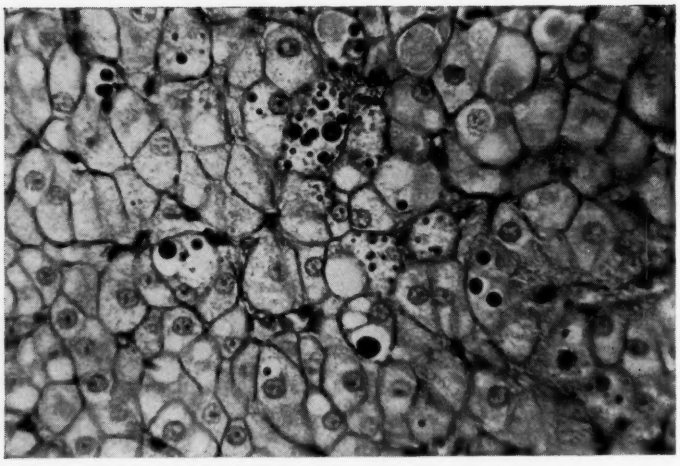

Fig. 4.—Adrenal of an STH-plus-MAD-treated sensitized rat (Group VIII). Accumulation of PAS-positive granules in the cortical cells and sharp delineation which gives them a "plant-cell-like" appearance. (PAS stain ×450, reduced ⅙.)

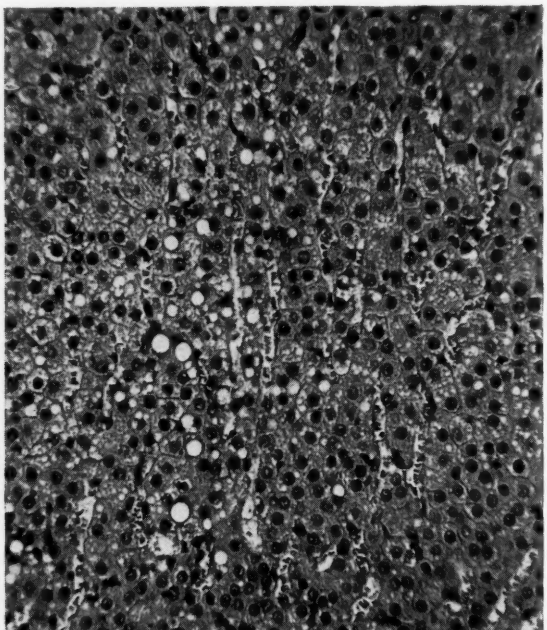

Fig. 5.—Adrenal of an STH-plus-MAD-treated intact rat (Group IV). Normal development of reticularis with many clear lipid vacuoles. (H & E stain ×250.)

Among the animals receiving STH alone, a relative atrophy of the reticularis cell layer was particularly striking, especially since the fasciculata cells were greatly developed and the total thickness of the cortex (including that of the reticularis) was in excess of the normal. This underdevelopment of the individual reticularis cells without diminution of the total width of the reticularis layer was quite evident, both in the intact and in the sensitized animals. Storage of large lipid granules was seen only in the animals receiving both STH and MAD (Figs. 4 through 6).

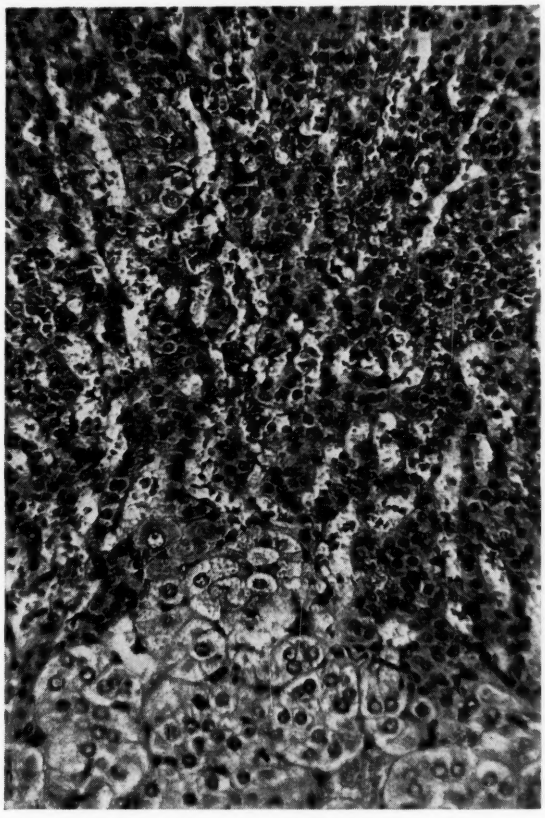

Fig. 6.—Adrenal of an STH-treated intact rat (Group III). Atrophic appearance of the reticularis cells in comparison with the previous photograph. (H & E stain ×250.)

The *thymus* underwent atrophy as a result of MAD, and hyperthrophy as a consequence of STH treatment, which is quite in agreement with the well-known "thymotrophic" effect of STH and the thymolytic action of various androgens. It is noteworthy, however, that the thymus involution produced by MAD could not be counteracted by simultaneous treatment with STH, even in the intact animals. In the sensitized rats, conjoint treatment with the two hormones actually aggravated the thymolytic action of MAD, presumably because of the great systemic toxicity of this hormone combination in rats so "conditioned." Systemic stressor agents of all kinds produce this thymolysis

through the stimulation of endogenous gluco-corticoid formation. It is noteworthy, however, that the adrenals of the sensitized rats were essentially of the same size in the group given STH alone and in that receiving MAD and STH; however, the former had the largest and the latter the smallest average thymus weights among all our groups. We emphasize this lack of correlation between thymic and adrenal development, because it shows again how misleading it may be to use the weight of the adrenal glands as the sole indication of their hormonal activity.

The weight of the *spleen* is listed merely to indicate that although this is largely a lymphatic organ, its development does not necessarily parallel that of the thymus. In this series the splenic weight was augmented by STH, both in the intact and in the sensitized series, but MAD alone caused no splenic involution and combined treatment with STH and MAD resulted in splenic weights which were even in excess of those obtained by STH alone.

The weight of the *hypophysis* was significantly depressed by MAD alone, both in the intact ($p < 0.01$) and in the sensitized ($p < 0.01$) animals, when compared with their respective untreated controls. A similar significant depression of pituitary weight was also observed when we compared the MAD plus STH-treated with the STH-treated-only intact ($p < 0.01$) or sensitized ($p < 0.01$) rats.

The *preputial glands* were weighed because they are a fairly accurate (although not very specific) indicator of androgenic activity. Their absolute and relative weight was greatly enhanced by MAD, and to a lesser extent also by STH (the latter observation confirms earlier findings of Selye[16]). This sexual effect was not increased by the sensitizing procedure.

Mesenteric *periarteritis nodosa* was not observed, either in the intact or in the sensitized rats, treated with MAD alone, at the comparatively low-dose level employed in this series of experiments. It should be remembered, however, that at higher dose levels, MAD had been shown to possess this activity.[10-12] STH notoriously differs from DCA and MAD in that it does not tend to produce mesenteric periarteritis nodosa even at high dose levels in sensitized rats.[3] In the present series only two animals in Group VIII showed some degree of mesenteric periarteritis nodosa, so that our material does not lend itself to any interpretation in this respect.

DISCUSSION

The experiments reported here clearly indicate that the same sensitizing procedure (unilateral nephrectomy and the substitution of 1 per cent NaCl for drinking water), which enhances the ability of mineralo-corticoids, such as DCA, to produce nephrosclerosis, polyuria, hypertension, and myocarditis in the rat, is also effective in "conditioning" the animals to the corresponding effects of MAD.

STH, which enhances all the previously mentioned actions of DCA, also aggravates the corresponding effects of MAD.

The development of the characteristic PAS-positive granules in the adrenals of MAD-treated rats was enhanced both by the sensitizing procedure

(unilateral nephrectomy and NaCl) and by simultaneous STH treatment; yet, in itself, neither the sensitization nor STH (nor STH plus sensitization) caused the development of such adrenal granules. It is perhaps noteworthy that the intensity of the PAS-granule development in the adrenals ran fairly parallel, under all conditions, with the renal and cardiac damage produced by the hormones and hormone combinations, except when STH was given alone.

SUMMARY

Overdosage with methylandrostenediol (MAD) produces a syndrome consisting of nephrosclerosis, myocarditis, periarteritis nodosa, hypertension, and polyuria, which is very reminiscent of that produced by mineralo-corticoids, such as desoxycorticosterone acetate (DCA). This effect is presumably mediated through the adrenals, since previous experiments have shown that it cannot be obtained after adrenalectomy.

The present experimental series demonstrates that sensitizing (or "conditioning") procedures which enhance the cardiovascular toxicity of mineralocorticoids, such as unilateral nephrectomy and a high-sodium intake or simultaneous treatment with somatotrophic hormone (STH), also enhance the corresponding toxic effects of MAD. These same "conditioning" procedures also augment the ability of MAD to induce the accumulation, within the adrenocortical cells, of PAS-positive granules. None of these procedures exert a significant effect upon such a rather typical sexual action of MAD as the stimulation of the preputial glands.

The thymic atrophy produced by MAD has not been counteracted under our experimental conditions by STH although the latter, in itself, induced marked thymus hypertrophy.

MAD caused a statistically significant involution of the hypophysis, both in intact and in sensitized rats, irrespective of whether they were simultaneously treated with STH or not.

The experiments give further support to the hypothesis which ascribes to STH the ability of sensitizing the body to the toxic actions of such steroids which can induce nephrosclerosis, hypertension, and cardiovascular lesions.

We are indebted to Dr. W. C. Murphy, of the Ciba Company Limited, Montreal, who supplied us with generous amounts of MAD for this work. We are also indebted to Miss Paulette Seguin, Miss Louise Derome, and Miss Marie Bélair for technical assistance and to Mr. Kai Nielsen and Miss Marie Langlois for the preparation of the chart and of the photographs.

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MECHANISM OF DIURNAL EOSINOPHIL RHYTHM IN MAN

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NORMAL people show a consistent diurnal variation in the number of circulating eosinophilic polymorphonuclear cells. In untreated adrenocortical insufficiency this fluctuation does not occur. Bliss, Sandberg, Nelson, and Eik-Nes[1] have demonstrated in normal subjects a diurnal rise and fall of plasma 17-hydroxysteroids. In patients with Addison's disease, however, they found not only low levels of 17-hydroxysteroids in plasma but also complete absence of any diurnal variation in the concentration of such material in plasma.

The purpose of the present study was to determine whether the normal eosinophil rhythm is (1) a reflection of the diurnal fluctuations in plasma corticoid concentrations or (2) a result of variations in the degree of "stress" existing in the body at different times of the day in the presence of reasonable amounts of circulating adrenal steroids. To prove the latter possibility would require the presence of an adequate but steady blood level of 17-hydroxycorticoids. Restoration of normal eosinophil variations in patients with Addison's disease by small amounts of cortisone administered evenly throughout the twenty-four hours of the day would assign to cortisone a "permissive" role. Conversely, restitution of normal eosinophil rhythm in Addison's disease by administration of a single, daily maintenance dose of cortisone, given at a time corresponding to the maximal plasma 17-hydroxysteroid levels in normal subjects, would indicate that these variations were directly related to sharp changes in the concentration of 17-hydroxysteroids in blood.

PROCEDURE

Total eosinophil counts were made every two hours over a twenty-four-hour period on six normal control subjects and on seven patients with Addison's disease. The normal subjects and the patients were permitted average daytime activity. All of the patients were maintained on desoxycorticosterone acetate, 2 mg. buccally after breakfast and after supper.

The following studies were carried out upon the patients with Addison's disease:

a. Seven patients received 2.5 mg. of cortisone orally every three hours for thirty-six hours. Eosinophil counts were performed every two hours during the last twenty-four hours.

b. Three patients remained on their customary schedule of oral cortisone therapy, namely, 10 mg. at 8 A.M., 5 mg. at 2 P.M., and 5 mg. at 8 P.M., and counts were made every two hours during a twenty-four-hour period.

c. Four patients received 25 mg. of cortisone orally at 6 A.M., and eosinophil counts were begun two hours later. No further cortisone was given to these individuals until 8 A.M. the following day.

d. Two patients received no cortisone but were studied in the same way.

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e. The effect on eosinophils of a four-hour ride on a stationary bicycle was studied on two patients receiving 2.5 mg. of cortisone orally every three hours. This schedule of administration of cortisone had been in effect for twenty-four hours before the beginning of the exercise period. It was assumed that this amount of activity would induce at least the same degree of "stress" (if not the same quality) as that occurring in the early waking hours.

Eosinophil counts were made on venous blood by Randolph's method.[2] A dilution factor of 1:10 was used, and triplicate counts were made on Neubauer hemocytometers. The total eosinophil count at 6 A.M. is taken as the point of reference for each patient. All subsequent values are expressed as the percentage change from the 6 A.M. value.

RESULTS

In Fig. 1 the thin lines represent the actual patterns and the heavy line the composite curve obtained from the counts of the six normal subjects. These curves show a maximal fall in total eosinophils between 6 A.M. and 2 P.M. followed by a gradual rise toward or above the 6 A.M. value in the early morning hours.

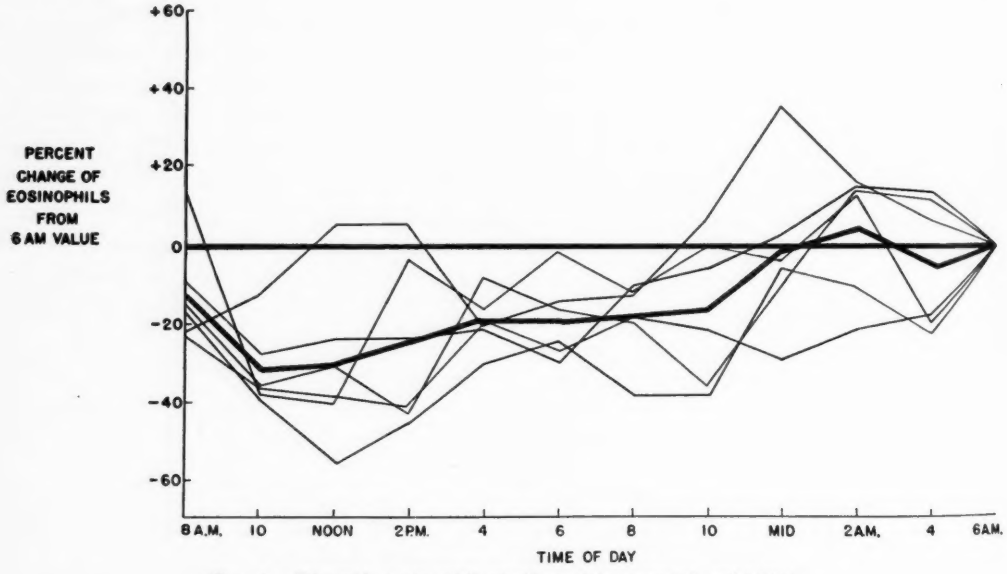

Fig. 1.—Diurnal eosinophil rhythm (six normal subjects).

Fig. 2 demonstrates eight individual curves and the composite one on seven patients with Addison's disease who received evenly administered cortisone. In five of the curves there is but slight variation throughout the entire twenty-four hours. The other three reveal a wide range of fluctuation, the extremes being a fall of 31 per cent and a rise of 56 per cent. In contrast, however, to the pattern in normal subjects there is no significant fall during the morning hours.

The composite curves exhibited in Figs. 1 and 2 are compared in Fig. 3. At 10 A.M. the curve obtained in normal subjects shows a maximal fall of 32 per cent from the 6 A.M. level. Thereafter it climbs slowly and attains its greatest height at 2 A.M. The curve from the patients receiving evenly administered cortisone shows no evidence of a morning fall, the 10 A.M. value being only 5 per cent below that for 6 A.M. Between 10 A.M. and 10 P.M. it rises moderately to a peak of 14 per cent above the 6 A.M. value at 4 P.M.

Shown, too, in Fig. 3 is the composite curve obtained from three patients with Addison's disease who received 10 mg. of cortisone at 8 A.M., 5 mg. at 2 P.M., and 5 mg. at 8 P.M. This curve is practically flat.

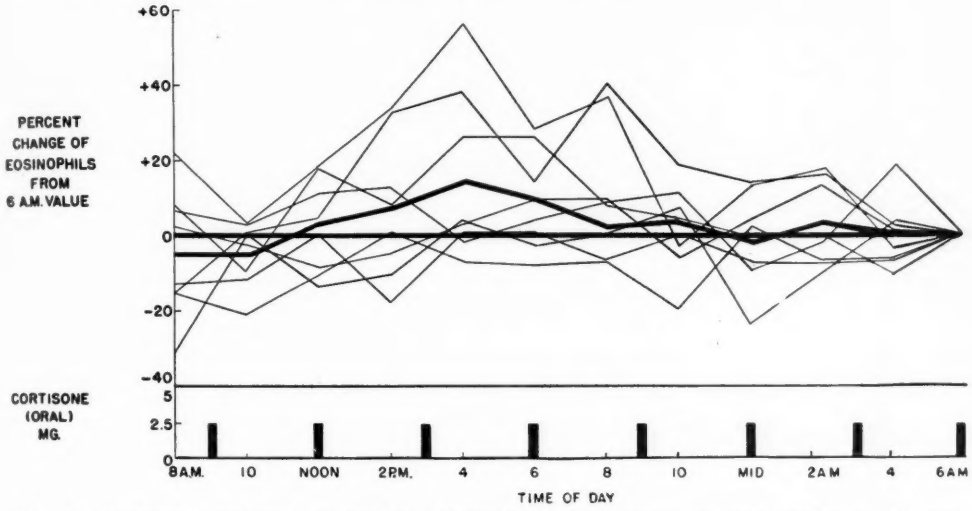

Fig. 2.—Eosinophil patterns in treated Addison's disease (seven patients receiving 2.5 mg. cortisone orally every three hours).

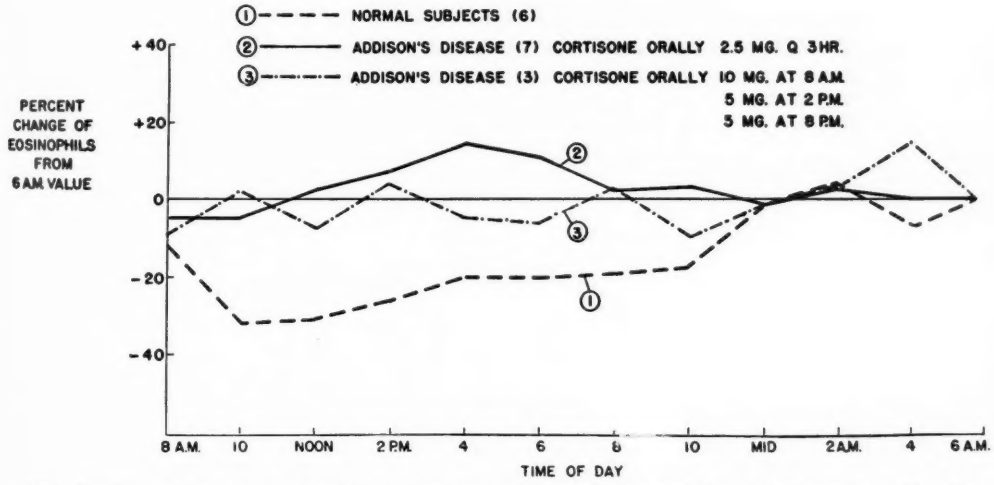

Fig. 3.—Comparison of eosinophil rhythm in normal subjects and in patients with Addison's disease.

Four patients were given a single oral dose of 25 mg. of cortisone at 6 A.M., and eosinophil counts were begun two hours later. Fig. 4 compares their composite curve with that of the normal subjects. The striking similarity is evident. The maximal fall is actually greater in the patients (46 per cent) than in the normal subjects (32 per cent). The lowest value occurs at noon in the patients and at 10 A.M. in the normal subjects. Both curves rise gradually during the evening and early morning hours.

The two patients who received no cortisone showed no significant fluctuation of the eosinophil count throughout the twenty-four-hour period. Table

TABLE I. ABSOLUTE EOSINOPHIL COUNTS

	NORMAL SUBJECTS						PATIENTS WITH ADDISON'S DISEASE															
							DCA (BUCCAL) 2 MG. B.I.D.		DCA (2 MG. BUCCALLY B.I.D.) PLUS ORAL CORTISONE													
									CORTISONE: 2.5 MG. EVERY THREE HOURS								10 MG. 8 A.M.	5 MG. 2 P.M.	5 MG. 8 P.M.	25 MG. AT 6 A.M.			
HOUR	J. R. K.	A.B.	J.H.	J. R. K.	H. K.	H. S.	T. K.	W. D.	F. H.	D. H.	H. P.	I. W.	E. W.	O. D.	T. K.	T. K.	E. W.	D. H.	I. W.	H. P.	E. W.	D. H.	F. H.
8 A.M.	167	150	253	167	235	375	353	511	112	174	289	78	67	414	417	252	79	356	162	644	62	218	185
10 A.M.	122	83	205	122	188	423	308	472	162	207	274	78	56	400	355	241	103	342	163	605	28	118	129
Noon	89	78	200	133	196	504	364	572	140	218	258	89	73	433	405	272	95	263	186	504	34	67	140
2 P.M.	111	128	189	111	196	504	342	477	134	274	269	72	67	438	461	305	106	353	186	582	50	101	157
4 P.M.	139	111	261	178	202	381	370	527	168	285	291	89	78	381	538	283	90	364	165	582	45	140	162
6 P.M.	151	131	239	161	179	409	342	487	157	235	310	89	78	403	438	279	73	370	195	577	62	174	196
8 P.M.	122	117	266	155	230	420	330	555	162	289	308	83	67	422	472	312	101	364	185	627	67	235	213
10 P.M.	122	133	255	122	241	510	358	472	173	246	263	89	65	433	356	257	78	325	184	610	67	375	235
Mid-night	189	128	228	172	263	650	308	561	123	235	294	83	62	355	390	311	84	358	200	588	62	420	207
2 A.M.	178	150	255	167	297	560	364	538	146	241	319	83	62	383	405	283	90	403	193	571	73	398	207
4 A.M.	155	105	266	216	291	510	297	538	168	213	287	83	56	461	333	287	118	308	238	700	67	465	185
6 A.M.	200	133	327	194	258	482	354	555	162	207	283	89	62	389	344	304	87	386	189	700	73	426	174

I presents the absolute eosinophil values obtained in all of the normal subjects and patients included in this study.

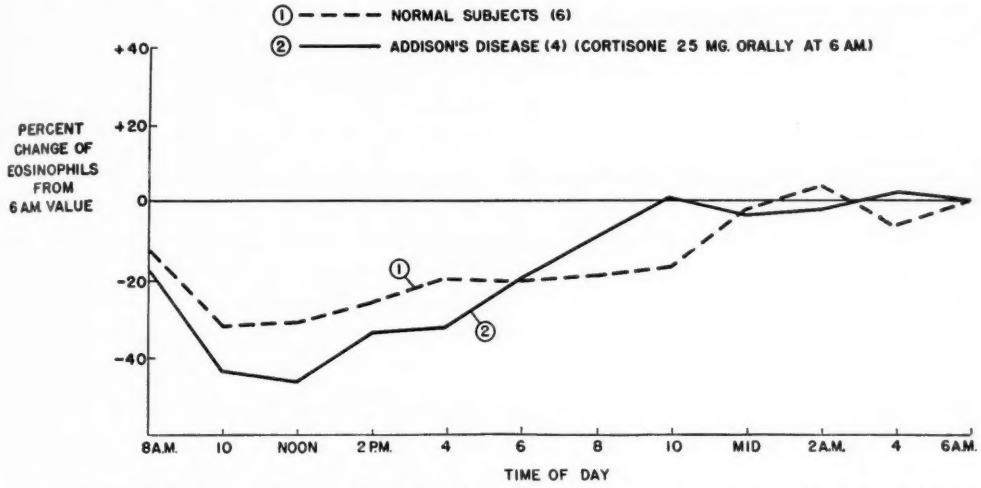

Fig. 4.—Comparison of eosinophil rhythm in normal subjects and in patients with Addison's disease.

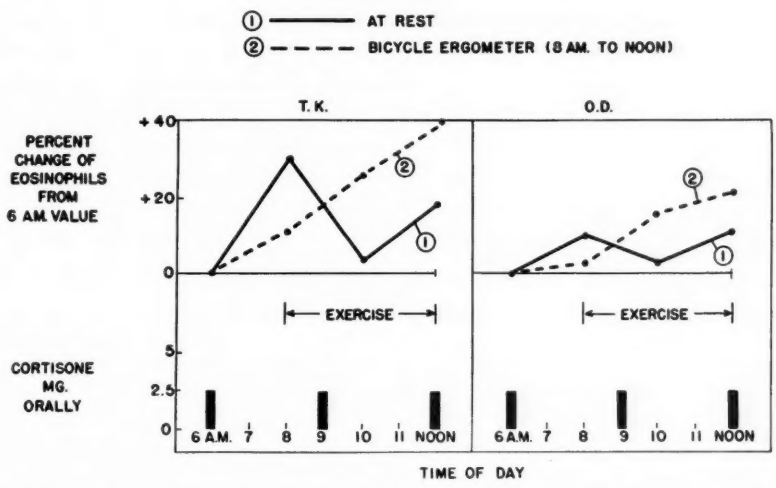

Fig. 5.—Effect of physical exercise on eosinophils in patients with Addison's disease receiving cortisone every three hours.

Fig. 5 compares the changes in eosinophils between 6 A.M. and noon (with and without exercise) in two patients with Addison's disease receiving 2.5 mg. of cortisone orally every three hours. It is clear that the superimposition of hard and prolonged exercise did not produce a decrease of circulating eosinophils.

DISCUSSION

A diurnal variation of the total eosinophil count in normal people has been established by Rud.[3] The pattern is characterized by night levels of eosinophils which are considerably higher than day levels, there being a sharp transition during the awakening hours of the morning from the nocturnal high to

the daytime low. Halberg, Cohen, and Flink[4] have reported that eosinophils of normal people fall at least 20 per cent between 6:30 A.M. and 9:30 A.M.

In order to permit comparison of our results with the previously described endogenous eosinopenia, 6 A.M. was selected as the point of reference in this study. Our control subjects demonstrated the typical diurnal fluctuation of total eosinophil levels as well as the morning endogenous eosinopenia.

Halberg[5] has shown that diurnal variation of eosinophils is absent (a) in patients with Addison's disease, (b) in totally adrenalectomized patients, and (c) in people with panhypopituitarism. To these may be added our observation that patients with adrenal insufficiency who receive adequate substitution therapy in a way which avoids wide fluctuations of blood levels of corticoids, also fail to exhibit the diurnal variation in the number of circulating eosinophils. This result, together with failure of superimposed severe exercise to decrease eosinophils, indicates that the normal diurnal eosinophil rhythm is not an example of the "permissive activity" of adrenal steroids. It suggests, too, that under normal circumstances a sharp *change* in the concentration of blood 17-hydroxycorticoid affects eosinophils more importantly than does the absolute concentration.

Bliss and associates[1] have shown that blood levels of 17-hydroxysteroids in man are highest in the morning and lowest during the late evening hours. In the present study, a diurnal eosinophil rhythm simulating the normal was produced in patients with Addison's disease when the total daily dose of cortisone was given orally at 6 A.M. This finding suggests that the normal daily variation in eosinophil levels is a reflection of diurnal fluctuations in the secretory activity of the adrenal cortices. The observation that eosinophil rhythm is restored in Addisonians by oral administration of a single 25 mg. dose of cortisone given in the morning, does not imply that the normal adrenal pours out most of its secretory product early and rests for the remainder of the day. It means only that early in the morning a sharp increase in the concentration of 17-hydroxycorticoids in the blood has occurred.

CONCLUSIONS

The normal diurnal rhythm of circulating eosinophils does not represent an example of the "permissive activity" of adrenal cortical hormone. The normal eosinophil rhythm reflects sharp changes in the intensity of peripheral activity of 17-hydroxycorticoids. The absolute number of circulating eosinophils is a poor index of 17-hydroxycorticoid concentration in body fluids. However, the changes in total circulating eosinophils, which are observed diurnally in normal persons, can be related to antecedent changes in the concentration of 17-hydroxycorticoids in body fluids.

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HEPATIC FUNCTION IN MULTIPLE MYELOMA

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HYPERGLOBULINEMIA is often found in such diseases as rheumatoid arthritis,[1] bacterial endocarditis,[2-4] sarcoidosis,[5, 6] systemic lupus erythematosus,[7-11] kala azar,[12] lymphogranuloma venereum,[13, 14] malaria,[15] and in other diseases.[2, 12, 16, 17] The frequently associated abnormality of the flocculation tests of hepatic function[18-20] is considered to represent alteration of plasma proteins rather than hepatic dysfunction.[19-23] Since extreme degrees of hyperglobulinemia occur in patients with multiple myeloma,[24-27] it is the purpose of this investigation to evaluate the incidence of apparent abnormality of liver function, particularly as measured by the flocculation tests, in patients with this disease.

METHODS AND PROCEDURE

Hepatic function tests were performed on twenty-two patients with multiple myeloma. The diagnosis was established by clinical, laboratory, and radiographic studies, and confirmed in all cases by bone marrow aspiration, and in seven patients by biopsy or at autopsy. The liver function tests used included the bromsulphalein test according to the modification of Mateer and associates[28] (5 mg. per kilogram of body weight with the estimation of retention at forty-five minutes). The degree of dye retention was measured photometrically with retention of 5 per cent or more considered abnormal. The thymol-turbidity test was done by Maclagan's method[29] as modified by Shanks and Hoagland,[30] with the upper limit of normal four units. The cephalin-cholesterol-flocculation test was performed according to the method of Hanger[31] with a reading of 3 or 4 plus at forty-eight hours considered positive. The total serum proteins were determined by the Biuret method with serum globulin precipitated by 26.86 per cent sodium sulfate solution. Zone electrophoretic separation of the serum protein fractions from ten patients was carried out by a method previously described.[32] Essentially this method consisted of applying the serum (usually 8 μl) to strips of Whatman No. 1 filter paper 1 inch wide. These strips had been equilibrated in the chamber with a barbital buffer, 0.05 M and pH 8.6. Electrophoresis was allowed to proceed for eighteen hours, the strips being suspended in the horizontal plane. At the end of that period the strips were dried at room temperature and stained with bromphenol blue. The background was destained by four washes with a dilute acetic acid solution, pH 3. The amount of protein present in each fraction of a serum sample was determined either by the dye elution method or by densitometry of the dye deposited on the strip.

RESULTS

Table I shows the results of liver function tests on the twenty-two patients with multiple myeloma. Abnormality of Bromsulphalein excretion (retention of 5 per cent or greater) was found in eight patients. The degree of retention,

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however, was minimal in all except two patients (W. T. and H. H.). In patient W. T. initial study performed during an episode of congestive heart failure revealed retention of 24 per cent of the dye. After successful treatment of the cardiac failure the degree of Bromsulphalein retention was reduced to 4 per cent as shown in the table. In patient H. H., who also showed retention of 24 per cent of the dye, heart failure was unremittingly present until death.

TABLE I. HEPATIC FUNCTION IN MULTIPLE MYELOMA

PATIENT	BSP (%)	T.T.	C.F.	T.P.	A	G	COMMENT
G. C.	2	0.7	3+	17.1	2.4	14.7	Positive cephalin flocculation tests; hyperglobulinemia; hypoalbuminemia
F. P.	6.5	0.3	3+	15.0	2.5	12.5	
O. C.	4.5	2.1	4+	12.2	2.3	9.9	
H. H.	24	20	3+	7.4	2.2	5.2	
A. H.	2	10.6	3+	9.6	3.0	6.6	Positive cephalin flocculation; hyperglobulinemia; normal albumin levels
N. W.	1	1.4	4+	8.7	3.8	4.9	
E. B.	6	1.1	4+	7.5	3.3	4.1	
R. M.	2.0	1.3	3+	9.8	3.4	6.4	
C. P.	4	1.1	2+	8.3	3.6	4.7	Insignificant cephalin flocculation; hyperglobulinemia; normal albumin values
C. Mc.	4	3.0	2+	7.8	4.0	3.8	
W. T.	4.0		0	9.4	3.4	6.0	
L. H.	5.6	5.0	1+	9.1	4.9	4.2	
E. A. B		0.4	0	8.1	3.1	5.0	
H. A.	5.5	5.1	0	8.3	4.3	4.0	
W. W.	8.5	0.3	0	8.0	2.6	5.4	Negative cephalin flocculation; hyperglobulinemia; hypoalbuminemia
R. L.	4.7	1.7	0	11.5	2.5	9.0	
C. H.		9.0	1+	15.0	1.5	13.5	
L. W.	6	0.7	2+	7.0	4.1	2.9	Normoproteinemia
G. S.	1.0	0.5	2+	6.5	4.5	2.0	
R. O. H			2+	5.0	3.0	2.0	
G. B.	7.5	1.0	0	6.5	4.0	2.5	
C. A.	1.7	1.0	0	6.5	4.4	2.1	

BSP = Bromsulphalein retention in the blood 45 minute (5 mg./kilo.).
T.T. = Thymol turbidity in Maclagan units.
C.F. = Cephalin flocculation.
T.P. = Total serum proteins.
A = Serum albumin.
G = Serum globulin.

Demonstrable abnormality of the plasma proteins occurred in seventeen patients of the group. Values over 8 Gm. per cent for total proteins were demonstrated in thirteen patients, and a globulin of 3 Gm. per cent or more occurred in seventeen patients.

Abnormal values for thymol turbidity were found in five patients, in two of whom the degree of abnormality was minimal. Cephalin flocculations of

TABLE II. FILTER PAPER ELECTROPHORETIC VALUES

	PER CENT AREA						PER CENT DYE ELUTION					
	ALBU-	GLOBULIN					ALBU-	GLOBULIN				
PATIENT	MIN	α_1	α_2	β	"M"	γ	MIN	α_1	α_2	β	"M"	γ
O. C.	29.8	3.7	9.6	11.0		46.0	18.3	3.1	5.8	5.3		67.5
L. H.	27.2	4.6	8.1	9.3		50.9						
E. A. B.	42.3	5.9	10.9	9.8		31.8	39.8	3.6	7.6	6.9		42.0
W. W.	35.9	5.1	12.0	10.7		36.4	22.6		15.0			62.4
C. H.	22.2	3.3	6.7	12.9		54.9	21.6	2.8	4.8	7.8		63.0
G. S.							62.0		30.8			7.3
G. B.	46.7	4.9	13.4	15.3		19.7	50.4	4.4	12.2	15.6		17.4
C. A.	61.0	3.9	12.1	12.0		11.0	66.6	5.1	8.5	11.6		8.2
R. M.	41.3	2.5	7.4	11.1	20.7	17.0	40.4	3.2	6.2	10.4	25.7	14.2
H. A.	55.5	2.8	8.9	10.5		22.3	50.1	3.7	6.5	9.2		30.5

3 to 4 plus appeared in eight patients in this study (Patients 1 through 8). Although all eight patients showed significant elevation of the serum globulin level and varying degrees of depression of the albumin fraction, similar abnormalities in other patients were accompanied by normal cephalin flocculation tests.

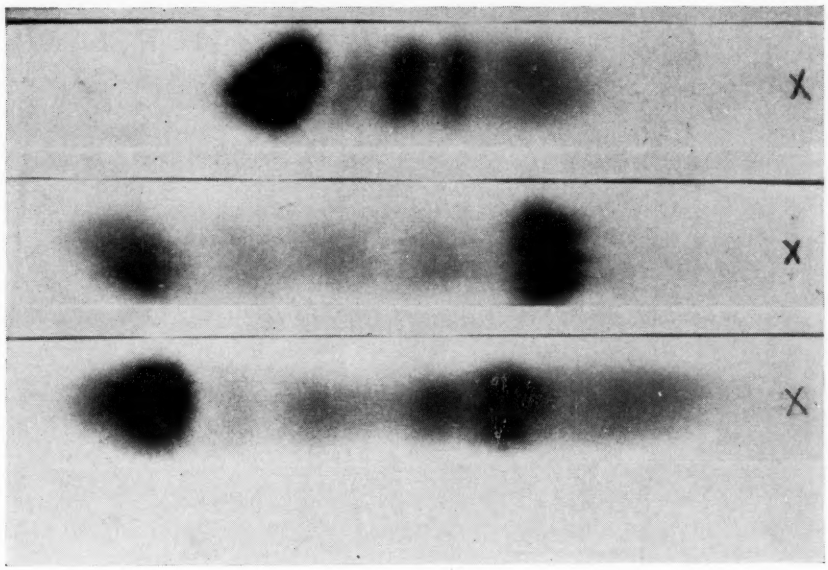

Fig. 1.—Filter paper electrophoretic patterns: (top) elevation of alpha$_2$ and beta fractions (G.B.), (middle) increase in gamma globulin levels (O.C.), and (bottom) "M" fraction between the beta and gamma globulin (R.M.).

The results of electrophoretic studies on sera from ten patients are shown in Table II. Significant changes in globulin fractions were found in all of the sera tested. Representative patterns are shown in Figs. 1 and 2. Altered globulins consisted of (1) increases in gamma globulin levels which were seen in five patients (L. H., O. C., C. H., E. A. B., and H. A.), (2) an abnormal component, the "M" fraction, between the beta and gamma globulin areas, which was found in one patient (R. M.), and (3) increases in more than one fraction which were found in four patients. The first two (C. A. and G. B.) showed an increase in the alpha$_2$ and beta globulin fractions. G. S. revealed an increase in the same fractions accompanied by a depression of the gamma globulin, and W. W. showed an increase of alpha$_2$, beta$_1$, and gamma globulin. As is seen in Table II, hypoalbuminemia occurred in seven of the ten patients.

Inconsistencies between the electrophoretic and the chemical studies of the serum proteins were noted in several instances, the most striking of which were in the apparently normoproteinemic group. A comparison of the electrophoretic patterns from Table II with the chemical fractionation reveals that while patients G. S., G. B., and C. A. were considered normoproteinemic according to the latter methods, electrophoretic studies revealed an increase in one of the globulin fractions.

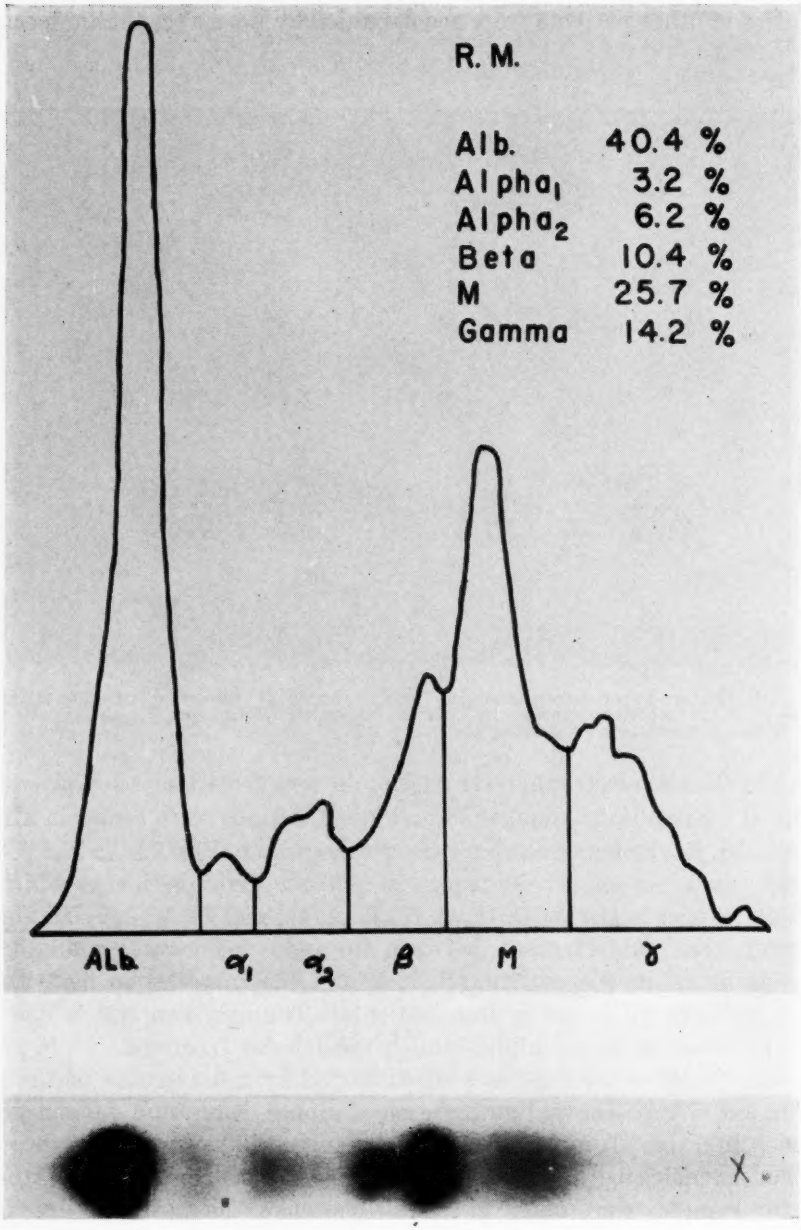

Fig. 2.—Zone electrophoretic patterns showing "M" component and area diagram obtained by direct densitometry of this strip.

DISCUSSION

Hyperglobulinemia, a frequently emphasized feature of patients with multiple myeloma,[24-27] occurred in seventeen of the twenty-two patients studied. The cephalin-flocculation test yielded abnormal results in only 36 per cent of the group and the thymol-turbidity levels were significantly elevated in only three out of twenty patients. In sarcoidosis, lupus erythematosus, subacute bacterial endocarditis, rheumatoid arthritis, and in a number of other diseases characterized by elevation of the serum globulin levels, abnormality of the thymol turbidity and the cephalin-flocculation tests is common.[18, 19, 20, 22, 33] It is, therefore, of interest that in multiple myeloma where globulin elevation is so frequent, the cephalin-flocculation and thymol-turbidity tests are more frequently normal than in other hyperglobulinemic diseases. Analysis of the nature of the globulin increase in multiple myeloma and in other hyperglobulinemic diseases is of interest in this connection. It has been demonstrated that the gamma globulins are most frequently elevated in multiple myeloma, the beta globulins infrequently, and the alpha globulins rarely.[34, 35] An abnormal "M" component has been occasionally described,[13, 36, 37] occurring electrophoretically between the beta and gamma globulins. Infrequently, more than one abnormal component is altered in patients with this disease.[13] In sarcoidosis, subacute bacterial endocarditis, lupus erythematosus, and rheumatoid arthritis, diseases associated with a higher incidence of abnormality of the flocculation tests, the most prominent and consistent globulin abnormality is elevation of the level of the gamma fraction.[5, 9, 13, 38, 39, 40, 41, 42] Thus the lesser incidence of abnormality of the flocculation tests in multiple myeloma may be due to the less frequent incidence of elevation of the gamma globulin. Consideration must also be given to the studies[43] suggesting that the serum globulins in patients with multiple myeloma may represent different proteins rather than an increase in normally occurring proteins. It has been suggested, although disputed, that apparent gamma globulin in some patients with multiple myeloma may include proteins which are considered abnormal or atypical proteins, having the same mobility but differing chemically from the normal gamma globulin.[42-46]

Thus, the observations[47, 48] that abnormality of the cephalin-flocculation test is related to gamma globulin elevation and potentiated by hypoalbuminemia may be an oversimplification when applied to multiple myeloma. Although most of the patients with positive cephalin-flocculation tests showed decreased levels of serum albumin, there was no consistent correlation between serum albumin levels and the results of these tests.

An interesting feature of the present study is the discrepancy between the incidence of abnormal thymol turbidity and positive cephalin-flocculation tests. Only three patients in this study revealed significant elevation of the thymol-turbidity value and only one had an accompanying positive cephalin-flocculation test. These results are in agreement with the observations[49, 50] that the cephalin-flocculation and thymol-turbidity tests depend on different changes in the plasma proteins. A similar "dissociation" between the frequently ab-

normal zinc sulfate turbidity and the infrequently elevated thymol-turbidity test in multiple myeloma has been reported by Maclagan and associates.[21]

Some differences between multiple myeloma and the other hyperglobulinemic diseases are shown in Table III. These differences have some clinical value. It would appear that marked hyperglobulinemia associated with normal thymol turbidity and negative cephalin flocculation is almost certainly not lupus erythematosus and probably not sarcoidosis but it is suggestive of multiple myeloma. Conversely, marked elevation of the thymol turbidity in an hyperglobulinemic patient makes multiple myeloma a less likely diagnosis.

TABLE III. INCIDENCE OF HYPERGLOBULINEMIA AND ABNORMAL HEPATIC FUNCTION IN MULTIPLE MYELOMA AND OTHER HYPERGLOBULINEMIC STATES

	ELEVATED SERUM GLOBULIN	NATURE OF GLOBULIN INCREASE	INCIDENCE OF ABNORMAL HEPATIC FUNCTION (%)		
			BSP	T.T.	C.F.
Multiple Myeloma	77%	Alpha, Beta, Gamma	42*	25*	36
Sarcoidosis[23]	67%	Gamma	54	54	48
Lupus Erythematosus[52]	88%	Gamma	38	96	68

*Usually minimal abnormality.

At the present time filter paper electrophoresis may be of considerable help in the diagnosis of multiple myeloma.[51] The tall narrow spikes, which are considered diagnostic of multiple myeloma, are unlike patterns obtained in other diseases of hyperglobulinemia, the latter revealing broader and wider complexes in the beta or gamma fractions.[24, 51] In the present study all of the electrophoretic patterns were abnormal even in the presence of normal chemical studies of the serum proteins and may therefore offer a clue to the diagnosis. The ease with which these patterns may be obtained by the use of filter paper electrophoresis warrants further consideration of this technique in the clinical laboratory.

A difference between the dye elution methods and the values obtained by densitometry of the dye deposited on the strip is seen in Table II. While it is not the purpose of the present report to evaluate the relative merits of these two methods for determining quantitatively the amount of protein present in each fraction, reproducible results can be obtained by either method. When the amount of protein in each fraction of the same strip was evaluated by the two methods, however, the agreement between them was less satisfactory. Studies of the possible reasons for these discrepancies are in progress in this laboratory. It is felt that, at the present state of knowledge, zone electrophoresis is suited better for the qualitative demonstration of abnormal protein components in serum than for the quantitative estimation of small differences in the amount of protein in each fraction.

With the exception of the two patients in congestive heart failure, Bromsulphalein excretion was abnormal in only six patients. The degree of retention in these six patients was minimal. Abnormally high values for Bromsulphalein retention may offer a clue to a complication occurring in multiple myeloma such as congestive heart failure, amyloidosis involving the liver, or other disease.

SUMMARY AND CONCLUSIONS

Tests of hepatic function were performed in twenty-two patients with multiple myeloma. Bromsulphalein excretion was found to be normal or only slightly abnormal in the majority of the group. In only two patients, both in congestive failure, was distinct abnormality of Bromsulphalein excretion demonstrated. The cephalin-flocculation test was abnormal in eight and the thymol-turbidity value elevated in five patients. Abnormality of plasma proteins was demonstrated in 77 per cent of the group. No correlation could be demonstrated between the degree of hyperglobulinemia and the results obtained with the flocculation tests. Electrophoretic studies revealed a variety of changes in the plasma protein fractions. The clinical value of these observations is discussed.

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INVESTIGATIONS ON THE AMINO ACID COMPOSITION OF A MACROGLOBULIN AND A CRYOGLOBULIN

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LITTLE is known about the amino acid composition of abnormal blood proteins. Apart from the difficulties experienced in assaying amino acids, the chief problem encountered in the study of these proteins is their isolation in pure form from the blood. Accordingly, only a few reports on amino acid analysis have been published.[1, 2] R. M. Hill and associates[1] described the assay of nine amino acids in a cryoglobulin which was isolated from the plasma in a case of multiple myeloma. Owing to the abnormal reactions of these proteins, they may sometimes be isolated by simple methods. The investigations reported in this paper concern two patients, one affected with macroglobulinemia (Waldenström[3, 4]), the other with cryoglobulinemia (Lerner, Greenberg, Watson, and Barnum[7-9]); the abnormal proteins had been isolated as stated later in the article.

MATERIALS AND METHODS

Case Reports.—

CASE 1.—The patient with macroglobulinemia was a 69-year-old man. For about 15 years he had been troubled by leg injuries which failed to heal completely. For over a year he had complained of fatigue and stiff numb fingers. There were no other complaints. On examination we found a marked cyanosis of the hands and feet. The patient was anemic. The entire body was covered with small, slightly elevated, brownish yellow patches, which were due to deposits of paramyloid in the skin, as was revealed by biopsy. Lymphomas, the size of which ranged from that of a hazelnut to that of a chestnut, were observed in the supraclavicular region, the axillae, and the inguinal regions. The liver extended for about 3 cm. below the costal margin, showing a firm, blunt border. The erythrocyte sedimentation rate had increased markedly, viz., 131 mm. after one hour (Westergren). Bence-Jones protein was found in the urine. X-ray examination showed no changes in the bones. The total serum protein was 9.58 Gm. per 100 ml., 5.8 Gm. of which was γ globulin. Electrophoresis showed a narrow high γ peak. When the serum was diluted with distilled water (1 to 15), 3.06 Gm. per 100 ml. of the protein was precipitated. Cooling of the serum at very low temperatures (refrigerator) resulted in the appearance of a turbidity which disappeared on heating. Puncture of the sternum and iliac crest yielded practically no material. Some very small specimens of bone marrow were obtained from a vertebral spine. These specimens were found to contain little but lymphoid cells of the type usually encountered in macroglobulinemia, and a number of tissue-mast cells. On ultracentrifugation a component with a sedimentation constant of 15.8×10^{-13} Svedberg units (S) (Fig. 4 and Table I) appeared in the serum.

CASE 2.—The patient with cryoglobulinemia was a 71-year-old man, who had been subject to dyspnea after exercise for about 8 months prior to hospitalization. For some weeks prior to hospitalization he had been liable to attacks of vertigo. He occasionally had slight nasal hemorrhages. He was not abnormally sensitive to cold. Examination of the body showed

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no pathologic changes with the exception of a moderately enlarged spleen, doubtful enlargement of the lymph nodes in the inguinal regions, and a marked anemia. When hospitalized the patient was occasionally seen to bleed diffusely from the gums, especially at night. The erythrocyte sedimentation rate, as in Case 1, was very high, viz., 153 mm. after one hour (Westergren). X-ray examination of the bones showed no changes. On centrifugation at room temperature the serum and plasma of the patient separated into two layers: an upper clear liquid layer and a lower turbid, markedly viscous layer. At 37° C. the viscous layer also became clear and more liquid, being precipitated again at about 28° C. The serum contained a total of 11.15 Gm. per 100 ml. of protein (Kjeldahl), 4.46 Gm. per cent of which was cryoglobulin. In paper electrophoresis the cryoglobulin at 37° C. reacted like a γ globulin. Dilution of the serum with distilled water did not result in turbidity as in the first patient. On ultracentrifugation two components with abnormally high sedimentation constants appeared (Fig. 4). Bone marrow biopsy again yielded very little material, which was found to contain lymphoid cells similar to those seen in the first patient and abnormal plasma cells (myeloma cells).

Accordingly there was not much difference between these two patients from a clinical point of view, but the reactions of the abnormal blood proteins were widely divergent.

RESULTS

Isolation of Proteins.—

Macroglobulinemia: As the patient's serum showed a marked euglobulin reaction in this case, a large sample of this serum (100 ml.) was diluted with distilled water (1 to 10), and a white precipitate was formed. After centrifugation the supernatant fluid was poured off, and the precipitate was readily dissolved by adding 100 ml. of a 0.9 per cent sodium chloride solution. From this solution the protein could again be precipitated by the same method of dilution with distilled water. This process of purification was repeated four times, after which the protein was dissolved in 0.9 per cent sodium chloride. The final solution was found to contain 1.41 per cent protein, with a Kjeldahl nitrogen content of 15.5 per cent.

Cryoglobulinemia: The cryoglobulin was isolated by the method of Lerner and Greenberg[7] of cooling the serum to 2° C. A flocculent precipitate was isolated by centrifugation at 0° C. This was redissolved in distilled water at 37° C. This process of purification was repeated three times. When the cryoglobulin had been dissolved three times in distilled water at 37° C., after about 24 hours, precipitation no longer occurred at 2° C. The addition of a small amount of 0.9 per cent sodium chloride immediately resulted in the appearance of a flocculent precipitate. When this process of purification had been repeated eight times, the protein was dissolved in distilled water at 37° C. This solution contained 1.24 per cent protein with a Kjeldahl nitrogen content of 15.7 per cent.

Properties of Proteins.—

Solubility: As the solubility of the two proteins in solutions of varying saline concentrations obviously was entirely different, some determinations of solubility were carried out. For this purpose equal amounts of each protein were added to equal quantities of varying concentrations of sodium chloride. Equilibrium was obtained by allowing the solutions to stand for 24 hours at

37° C., with occasional shaking. After filtration at 37° C. the protein content of the supernatant fluid was determined by assaying the nitrogen content.

The solubility of the protein at varying ionic concentrations differs markedly at 37° C. (Fig. 1). Macroglobulin (the solution used was approximately 1.41 per cent) was insoluble in 0.000 to 0.024 M of sodium chloride, while the protein dissolved when the saline concentration was increased from 0.024 to 0.090 M. When the concentrations were increased to an even greater extent (up to 0.540 M NaCl), no precipitation occurred. The cryoglobulin, added until a final concentration of 1.24 per cent was obtained, remained in the dissolved state when distilled water was used as a solvent. When approximately 0.001 M of sodium chloride was added, about 50 per cent was precipitated, while in 0.003 to 0.008 M of sodium chloride only 12 per cent of the protein was soluble. Increasing the saline concentration resulted in a slowly increasing solubility, which was 100 per cent at 0.075 M of sodium chloride (Fig. 1).

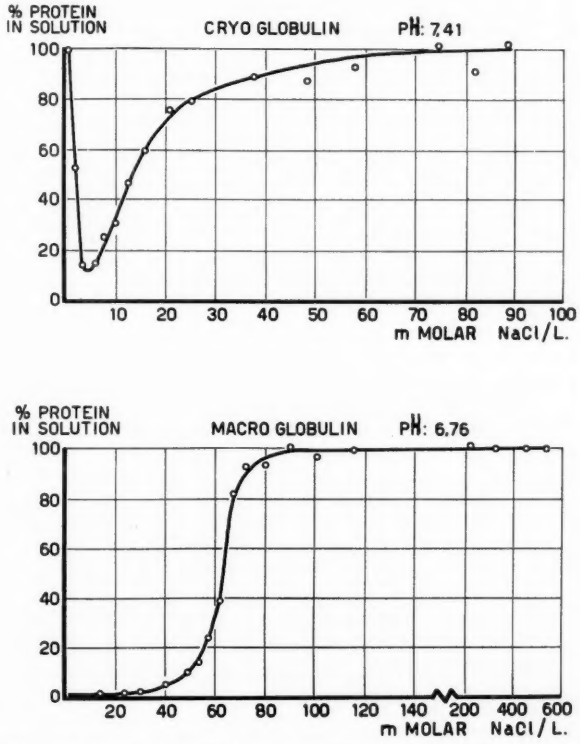

Fig. 1.—Protein solubility in solutions of varying concentration of NaCl at 37° C.

Absorption spectrum: The ultraviolet absorption spectrum of the two proteins was determined with a Beckman quartz spectrophotometer with 1 cm. cuvettes at room temperature. The macroglobulin was dissolved in 0.145 M of sodium chloride and the cryglobulin in 0.080 M of a sodium chloride solution. The two proteins were compared with solutions of commercial human γ globulin (in 0.140 M of sodium chloride) and of commercial human albumin (in

0.140 M of sodium chloride). As shown in Fig. 2 the absorption spectra of the two abnormal proteins were approximately identical with the curves obtained by studying the γ globulin, although there were some minor differences.

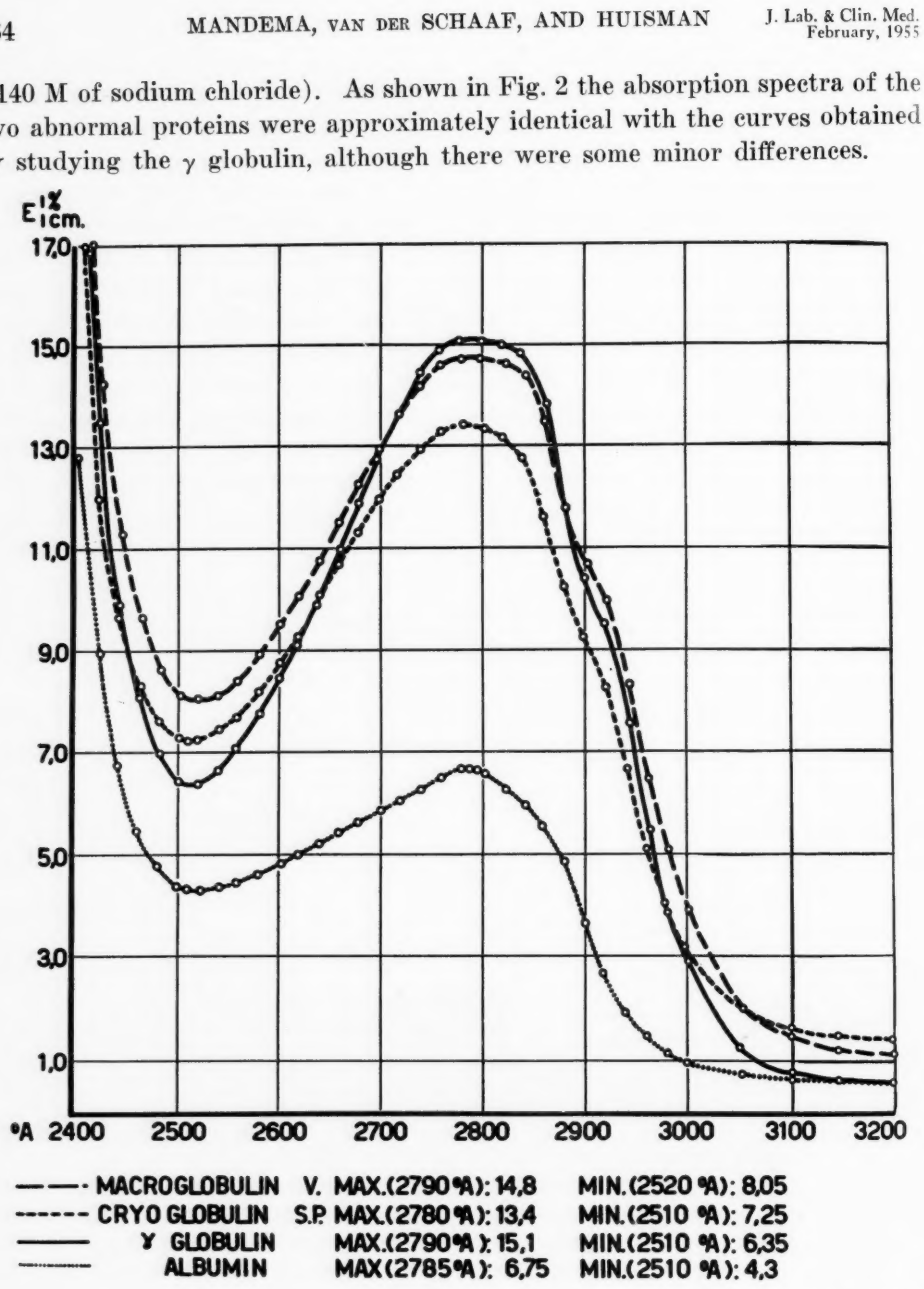

Fig. 2.—Comparison of the ultraviolet spectral absorption of the isolated proteins and of globulin and albumin.

Electrophoresis: The electrophoretic pattern of the macroglobulin in the serum and in pure solutions was studied by the Tiselius technique, whereas the cryoglobulin solutions could only be studied by paper electrophoresis at 37° C. The results given in Fig. 3 show that both sera contained a large amount of γ globulin. The isolated macroglobulin seemed to migrate with a symmetrically shaped boundary, but another component with a greater

mobility was present in a proportion of about 3 per cent, while at the same time a very small quantity (< 2 per cent) of a third component was found. The cryoglobulin solution studied by paper electrophoresis at 37° C. contained only one component, migrating like a γ globulin (Fig. 3).

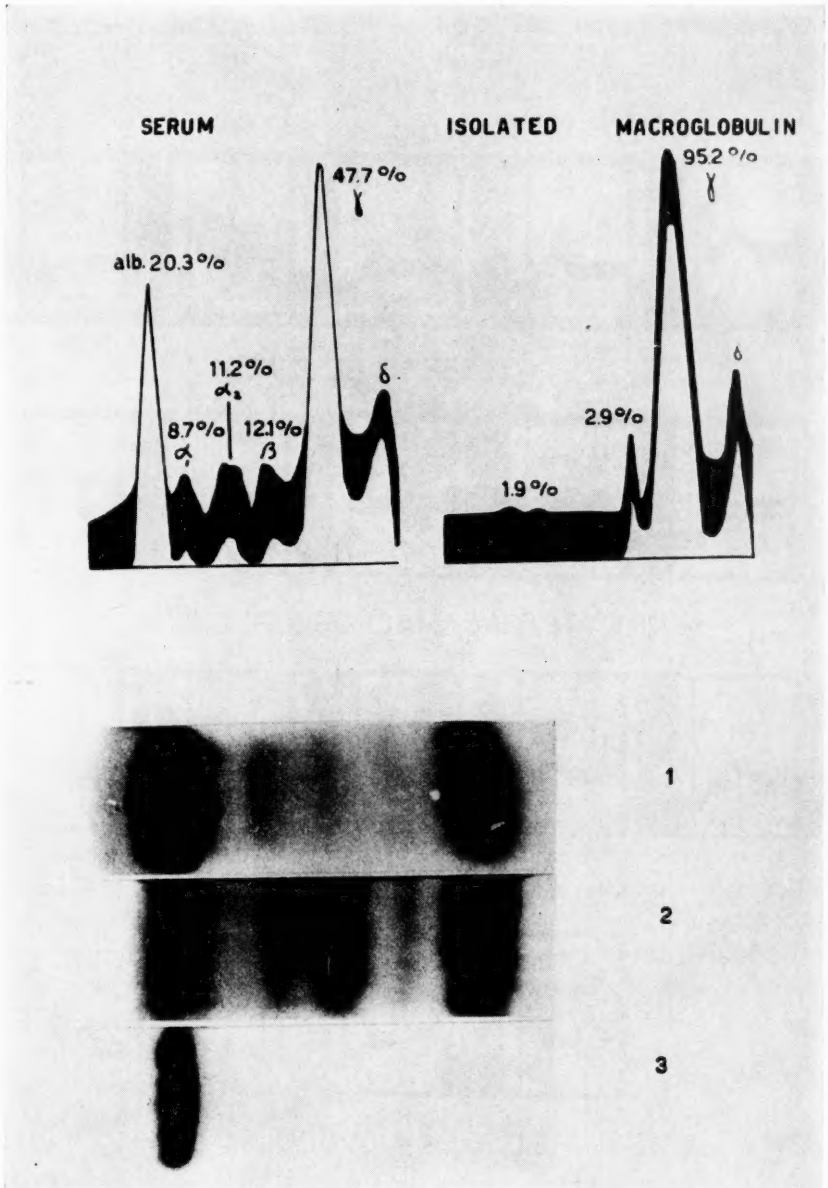

Fig. 3.—The electrophoretic pattern of the macro- and cryoglobulin. The macroglobulin was studied by the Tiselius technique and the cryoglobin by paper electrophoresis at 37° C. *1*, Electrophoresis of the precipitate isolated from the serum at 18° C. *2*, Electrophoresis of the supernatant serum. *3*, Electrophoresis of the purified protein.

Ultracentrifugal studies: These experiments, which were carried out by Dr. H. Reerink of the Van't Hoff Laboratory, Utrecht, showed that both sera contained components with high sedimentation rates. The fact should be mentioned that the cryoglobulin solution had to be studied at 30° C. in order to prevent precipitation of the protein at lower temperatures. The results given in Fig. 4 show that the macroglobulin solution still contained a small quantity of a com-

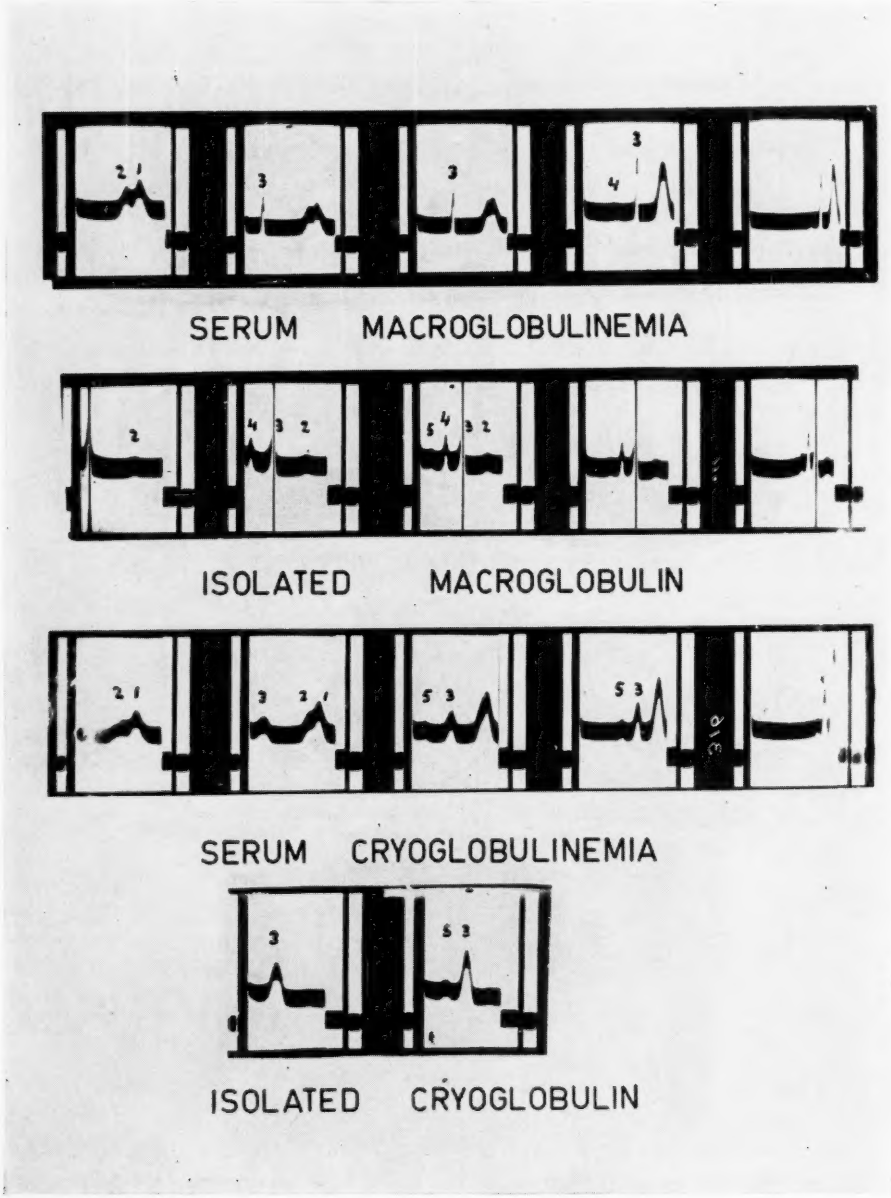

Fig. 4.—Sedimentation diagrams of the sera and the isolated proteins. The figures in the diagram correspond to those of Table I.

ponent with a sedimentation constant S_{20} of 6.9×10^{-13} (a normal globulin?), whereas most of the protein had a sedimentation rate identical to that determined in the serum. The presence of the two other components with still higher sedimentation rates (Table I) was due to an interaction of the protein molecules rather than to impurities. The cryoglobulin solution contained a large amount of a component with a sedimentation constant of 19.2×10^{-13}, while another, with a much higher sedimentation rate, was also present. No impurities could be detected.

TABLE I. THE SEDIMENTATION CONSTANTS ($S_{20} \times 10^{-13}$) OF THE PROTEINS IN THE SERA AND IN SOLUTIONS IN A CASE OF MACROGLOBULINEMIA AND A CASE OF CRYOGLOBULINEMIA

	1	2	3	4	5
Macroglobulinemia serum	4.0	7.0	15.8		28.0
Isolated protein	-	6.9	14.6	22.7	28.0
Cryoglobulinemia serum	4.0	6.4	16.9		29.0
Isolated protein	-	-	19.2		30.9

Amino Acid Composition.—About 300 mg. of protein was hydrolyzed in 200 ml. of 6 N HCl for 48 hours at 100° C. After the hydrochloric acid had been removed in vacuo, the solution was finally reduced to a small quantity by concentration. The amino acids in this mixture were separated and assayed by ion exchange chromatography, using the technique as described by Moore and Stein.[14] The results of the analysis are given in Table II and compared with the amino acid composition of γ globulin[10] together with the values found by Hill and associates[1] for an isolated cryoglobulin, and the analysis of a Bence-Jones protein by Ägren.[11]

DISCUSSION

When Dent and Rose[12] studied the Bence-Jones protein isolated from the urine of a patient with multiple myeloma by paper chromatography they found no methionine, while recently Gunnar Ägren[11] reported the amino acid composition of Bence-Jones protein isolated from both the urine and a tumor mass of a patient with multiple myeloma, using microbiologic methods. He found a content as low as 0.4 per cent in both cases.

A possible relationship between Bence-Jones protein and abnormal serum proteins is quite unknown. It may be that the Bence-Jones proteins are formed by the myeloma cells, or originate from abnormal blood proteins. The results of the amino acid analysis of the abnormal proteins reported in this paper show that the amounts of the majority of the amino acids are about the same as those described for normal γ globulin. The methionine contents, especially, are completely identical and about three times the quantities given for Bence-Jones protein. Therefore, it is unlikely that this protein is formed by the breakdown of abnormal blood proteins. The fact should be borne in mind, however, that electrophoretic and immunologic studies suggest that there are various Bence-Jones proteins, while the abnormal blood proteins may also differ in many respects. This indicates that a complete comparison between our values and the results of amino acid

analysis of Bence-Jones protein, as reported by Ägren, is impossible. Unfortunately we did not succeed in isolating the Bence-Jones protein in pure form from the urine of the patient affected with macroglobulinemia. In this connection the papers of Putnam and Hardy[13] are of importance. By using glycine labeled with N^{15} or C^{13} these authors were able to demonstrate that in two patients with abnormal blood proteins and Bence-Jones proteinuria the rate at which Bence-Jones proteins were produced was independent of that at which the serum proteins were formed.

TABLE II. THE AMINO ACID COMPOSITION OF THE ISOLATED PROTEINS

AMINO ACID	MACROGLOBULIN	CRYOGLOBULIN	γ GLOBULIN[10]	CRYOGLOBULIN (HILL[1])	BENCE-JONES PROTEIN (ÄGREN[11])
Hydroxyproline	0	2.0	0		
Aspartic acid	10.7	8.6	8.8		6.1
Threonine	8.7	9.25	8.4	5.88	9.5
Serine	8.9	8.4	11.4		16.7
Glutamic acid	11.2	12.05	11.8		16.6
Proline	1.6	9.7	8.1		6.4
Glycine	4.2	4.0	4.2		2.3
Alanine	5.1	4.45			6.0
Cystine		2.25	2.4		3.8
Valine	10.2	7.7	9.7	9.60	11.0
Methionine	1.3	1.05	1.1	0.90	0.4
Iso-leucine	3.3	3.3	2.7	2.13	4.2
Leucine	9.2	8.8	9.3	8.70	10.6
Tyrosine	4.5	5.0	6.8		7.3
Phenylalanine	6.4	5.2	4.6	3.99	2.7
Tryptophane			2.9	1.59	3.9
Histidine	1.5	2.05	2.5	1.96	1.0
Lysine	7.1	5.6	8.1	7.28	4.4
Arginine	7.0	6.4	4.8	5.56	5.7
Total	101.9	105.8	106.5		118.6
Per cent N calculated	92.0	94.0	95.0		95.5

In view of the data given in Table II, it might be of interest that the proline content of the macroglobulin is very low, whereas the cryoglobulin contains a slightly increased percentage of this amino acid. Moreover, hydroxyproline was present in this protein. This amino acid was not found in normal blood proteins.

SUMMARY

The macroglobulin was isolated in a case of macroglobulinemia (Waldenström) by diluting the serum with distilled water. The cryoglobulin was isolated in another patient with cryoglobulinemia by cooling the serum.

The method of purification of the two proteins is described.

The solubility in saline solutions was studied. The ultraviolet absorption spectra of the two abnormal proteins were compared with the spectrum of commercial human γ globulin and human albumin. The electrophoretic patterns of the sera and the protein solutions are given. Both sera contained proteins with high sedimentation rates as determined by ultracentrifugation.

The macroglobulin solution also contained a very small amount of a protein with a normal sedimentation rate, whereas no impurities could be detected in the cryoglobulin solution when this method was used.

Finally the amino acid composition of the two proteins was assayed by ion exchange chromatography.

The methionine content, as compared to normal human γ globulin, was normal.

Bence-Jones protein, on the other hand, is reported to contain no, or only very little, methionine. This result is at variance with the hypothesis that Bence-Jones proteins originate from abnormal blood proteins. The proline percentage was very low in the macroglobulin, but showed a slight increase in the cryoglobulin. In addition, hydroxyproline, an amino acid not to be found in normal blood proteins, was present in the cryoglobulin.

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EFFECT OF CHRONIC INJECTION OF A HEPARIN COMPLEX ON AORTIC ATHEROSCLEROSIS IN CHOLESTEROL-FED CHICKENS

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THE dramatic effect of heparin injection on the clearing of alimentary lipemia and on the redistribution of plasma lipoprotein fractions in mammals[1-4] has invigorated the field of atherosclerosis research. Although precise relations are not yet firmly established in mammalian test animals or in man, it is of interest to examine the effect of chronic injection of heparin in chickens since this species is so widely used in atherosclerosis research. We present, therefore, an account of our observations concerned with the effect of heparin on the incidence and severity of atherosclerosis in the cholesterol-fed chicken.

METHODS

White Leghorn cockerels were used in all experiments. The chickens were fed a standard starter mash, either alone or supplemented with 2 per cent cholesterol (Merck, U.S.P.) and 5 per cent Wesson oil. The incidence and severity of aortic atherosclerosis was estimated by the criteria used by Horlick and Katz,[5] the lesions being graded on a 0 to 4 basis. Consistency in grading the fresh material was tested by confounding the fixed specimens and regrading.

Plasma cholesterol determinations were made by the method of Sheftel.[6] Lipid phosphorus was estimated by the method of Fiske and Subbarow[7] and total lipids by the alcohol-ether extraction procedure.

The method of Gofman and associates[8] was employed in the isolation and analysis of the serum lipoproteins. Minor modifications were introduced, the most important being the use of plasma rather than serum in order to minimize the amount of blood withdrawn from each chicken. Two to four milliliters of plasma was diluted to 11 ml. with saline solution so prepared that the final solution density was 1.063 (9.2 wt. per cent NaCl). The solution was centrifuged in a preparative cell at 180,000 g. The top 1.5 ml. was pipetted off and run in the analytic ultracentrifuge at 250,000 g. Results were obtained for several classes of flotation velocities, that is, for molecules of S_f 3 to 10, 10 to 20, 20 to 30, 30 to 100, and 3 to 100 or 100 to 400, where S_f is a unit of flotation velocity equaling 10^{-13} cm./sec./dyne.

Four groups of 8-week-old White Leghorn cockerels (12 birds per group) were placed on the high cholesterol diet. Two of the groups received a daily injection of 8 mg. butacaine-heparin complex* (equivalent to 3 mg. heparin) intramuscularly for 8 weeks. The other two groups, both on the high cholesterol diet, but receiving no injections, served as control groups. One group each from the injected and noninjected groups was used for plasma studies. The remaining two groups were not bled at any time during the course of the experiment. Statistical comparison of the necropsy data on the bled and nonbled groups revealed no significant difference between the results. Therefore, the data were pooled.

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*The buctacaine-heparin complex was prepared by Dr. Dale Goldsmith, Organic Biological Chemistry Division, Merck and Company, Inc.

RESULTS

Table I summarizes the necropsy data. The pathologic observations were limited to an estimation of the incidence and severity of thoracic aorta atherosclerosis. Contrary to expectations, the incidence of atherosclerosis in the cholesterol-fed control groups was less than 100 per cent. The factors which are responsible for the difference between our over-all incidence of 67 per cent and the 100 per cent reported by Rodbard and associates[9] have been discussed elsewhere.[10] Although the differences between the mean averages of incidence and severity suggest some inhibitory action of heparin on the production of experimental atherosclerosis, the differences, when viewed statistically, are not great enough to be significant ($P = 0.2$). Thus, it appears that chronic heparin injection does not significantly reduce the severity and incidence of aortic atherosclerosis in the chicken.

TABLE I. INCIDENCE AND SEVERITY OF AORTIC ATHEROSCLEROSIS IN CHOLESTEROL-FED,* HEPARIN-TREATED† CHICKENS

GROUP	NO.	TREATMENT	INCIDENCE (PER CENT)	SEVERITY INDEX 0-4*	
				INC. NEGATIVES	EXC. NEGATIVES
I + II	24	Control	67	1.12	1.68
III + IV	21	Heparin treated	48	0.62	1.18

*2 per cent cholesterol + 5 per cent Wesson oil eighth to sixteenth week.
†Daily intramuscular injection 8 mg. butacaine-heparin eighth to sixteenth week.

Plasma samples were obtained from the same five chickens in each of a control and heparin-treated group at two-week intervals. Table II shows the result of the plasma lipid analyses on these samples. All plasma lipid fractions increased promptly and progressively (except the phospholipid at the eighth week) in the control group. In the heparin-treated group, however, all plasma lipid fractions, after showing an increase during the first four weeks comparable to that observed in the control group, declined significantly. The decrease in the concentration of total cholesterol from 1,031 mg. per cent at four weeks to 684 mg. per cent at eight weeks is particularly striking.

TABLE II. AVERAGE PLASMA LIPID CHANGES INDUCED BY DAILY INJECTIONS OF BUTACAINE-HEPARIN IN FIVE CHOLESTEROL-FED CHICKENS

WEEKS OF TREATMENT	TOTAL CHOLESTEROL (MG. PER CENT)	FREE CHOLESTEROL (MG. PER CENT)	TOTAL LIPID (MG./C.C.)	PHOSPHOLIPID (MG. PER CENT)
I. Heparin Treated				
0	190 (168-236)*	26	15.6	174 (164-200)*
2	1,006 (538-1880)	226	26.4	261 (167-404)
4	1,031 (475-1820)	206	25.6	278 (146-381)
6	788 (471-1345)	156	20.1	211 (160-308)
8	684 (225-1340)	160	21.1	189 (103-318)
II. Controls: No Heparin				
0	193 (168-212)	30	15.9	184 (165-207)
2	889 (689-1138)	202	25.0	246 (195-287)
4	968 (615-1379)	239	27.0	284 (181-404)
6	968 (706-1743)	246	28.0	307 (214-395)
8	1,084 (540-1960)	254	28.6	258 (167-402)

*Range.

The results of the lipoprotein analysis show so much individual variation that exhaustive treatment of these data is not warranted. Cholesterol feeding, as expected, increased the concentration of lipoproteins in all S_f classes. Heparin treatment apparently had little effect on the concentration of lipoproteins in the S_f 3 to 10 class. The S_f 10 to 20 lipoprotein concentration in four heparin-treated chickens declined from an average of 60 mg. per cent to 40 mg. per cent over the eight-week period of treatment, but the concentration increased from 70 to 180 mg. per cent in the fifth chicken. The control birds, except one, showed increasing concentrations of the S_f 10 to 20 molecules. The average concentration in these four birds increased from 60 mg. per cent to 127 mg. per cent over the eight-week period. The fifth bird exhibited a decline in the S_f 10 to 20 class from 70 to 48 mg. per cent. The change in the concentration of S_f 30 to 100 molecules was most inconsistent in both the heparin-treated and control groups. While the concentrations in both groups were elevated above that found in birds on a stock diet, the week-by-week changes were most erratic and no special trend could be seen.

DISCUSSION

It is quite apparent that we have failed to demonstrate convincingly any inhibitory effect of chronic heparin injection on the incidence or severity of aortic atherosclerosis in the chicken. Such a failure does not deny the possibility that heparin has effects on the metabolism of lipids and lipoproteins in this species, however. The decrease in total plasma cholesterol and other lipid fractions after four weeks of heparin injection seems real and is not explained on the basis of impaired food consumption or loss of body weight since no differences in these factors were noted. Furthermore, our data on changes in lipoprotein pattern, although inadequate for making certain conclusions, do suggest that chronic heparin injection tends to prevent the rise of S_f 10 to 20 class molecules which was observed in the cholesterol-fed chickens.

The failure to obtain clear-cut results may be due to the difficulty in maintaining an adequate blood level of heparin. It was our thought that a slowly absorbed heparin complex such as the butacaine-heparin preparation would maintain a high enough blood level to produce results and yet not complicate the experiment by introducing difficulties due to hemorrhage. It now seems clear that a better way of subjecting experimental animals to the constant influence of heparin must be devised in order to solve this problem satisfactorily.

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OBSERVATIONS ON THE ORIGIN OF ASCITES FROM EXPERIMENTAL HEPATIC CONGESTION

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UNTIL recently it was frequently stated that ascites from primary liver disease or hepatic congestion resulted from portal hypertension and consequent peritoneal transudation. Recent studies of experimental ascites,[1] however, have suggested that the liver is the principal source of proteinous ascitic fluid when critical congestion of the organ occurs. Our present observations concern the latter mechanism.

METHOD

Seven mongrel dogs of 20 to 30 pounds each were selected for the experiments. These animals were subjected to surgical constriction of the thoracic inferior vena cava by the technique of McKee and his associates.[2] The details of the procedure need not be repeated; however, it is important that adequate exposure of the thoracic inferior vena cava was attained by entering the chest through the sixth right interspace so that a small aluminum band could be applied to the inferior vena cava accurately. The band was lined with a covering of reactive Cellophane so that constriction of the inferior vena cava was produced slowly by fibrosis and eventually approximated 60 to 70 per cent of the original luminal diameter. The abdomen was not incised during the procedure. Penicillin (400,000 units) and streptomycin (0.5 Gm.) were given daily for two or three days postoperatively.

Recovery from the operation was complete in about one week. The usual kennel ration was then supplemented by the daily administration of commercial canned dog food (1 pound) mixed with 5 to 10 Gm. of salt. The mixture was readily taken by the animals. Massive ascites generally occurred within one week on this regime.

When ascites formation was well established, samples of abdominal fluid and blood plasma were obtained for protein analysis. Thereafter, the ascitic fluid was removed by paracentesis and laparotomies were performed on the animals. Under Nembutal anesthesia and positive pressure breathing the abdomen and lower thoracic cage were opened by midline incision for wide exposure of the liver. In several animals a polyethylene tube was inserted into a splenic venous radicle and advanced into the portal vein near the hilum of the liver. Five to eight milliliters of 5 per cent Evans blue dye in saline were injected through the catheter (or in some animals by direct puncture of the portal vein) to color the hepatic lymph for visualization of the lymphatics. The tube was retained and attached to a simple water manometer for measurement of portal pressure. Samples of liver lymph were obtained by cannulation of one of the extrahepatic lymphatics with a

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fine polyethylene tube.[3] Fluid forming on the liver surface (hereafter referred to as "capsular liver fluid") was allowed to drain onto nonirritative Cellophane sheets placed under the left lobe. Fluid samples were then removed with capillary pipettes. In some cases capsular liver fluid collection was facilitated by obtaining droplets of fluid directly from the liver surface with fine pipettes. Inferior vena caval pressure was measured at the level of the hepatic veins by means of a polyethylene tube introduced through a femoral vein, and attached to a water manometer. Arterial blood pressure was recorded by cannulation of a femoral or common carotid artery and the use of a mercury manometer. In some experiments, a loop of small intestine was placed in a nonirritative Cellophane bag for collecting specimens of peritoneal fluid. Although care was taken to avoid interference with the blood supply of the loop, there was always the question of undetected kinking of the vessels. Therefore, we relied principally on inspection to detect transudation from the various serous surfaces.

All fluid specimens were collected in oxalated test tubes. Blood counts were done on the fluids before centrifugation. Protein determinations were made on all specimens using the ammonium chloride precipitation method described by Weichselbaum.[4]

RESULTS

After effective constriction of the supradiaphragmatic inferior vena cava had been achieved, the animals developed massive ascites when the regimen included increased salt intake as described. One dog required further constriction of the inferior vena cava before ascites occurred. As ascites formed, the dogs exhibited variable degrees of muscular wasting. Extensive venous collateral channels were noted on the abdominal wall. Other observers have found that similar experimental animals may not show persistent portal hypertension.[5, 16] However, at laparotomy these dogs had portal pressures averaging 18 cm. of water, in contrast to the average portal pressure of 9 to 15 cm. of water in *normal* anesthetized dogs at laparotomy.[18-20] Inferior vena caval pressure, distal to the point of constriction and at the level of the hepatic veins, averaged 12 cm. of water, a value of twice the normal caval pressure in anesthetized dogs. Arterial blood pressures were within normal limits during the experimental procedures.

The livers of the dogs were moderately enlarged, turgid, and of purple hue. Small patches of fibrinous material were noted over the capsular surfaces, and small fibrinous adhesions (some vascularized) extended from the liver to the abdominal wall. The most striking observation was the presence of innumerable droplets of fluid over the entire hepatic surface. These droplets constantly coalesced and trickled from the liver into the peritoneal cavity. Some of this fluid collected in the spaces between the lobes and rapidly clotted. It was bluish in color from the dye. Because of the anatomic position of the liver it was not possible to measure the total quantity of fluid escaping from the surface. Nevertheless, fluid formation was so rapid that it was often possible to collect 5 to 10 ml. within a very few minutes. This abundant formation of capsular liver fluid persisted throughout the experiment (often five to six hours) at an apparently unabated rate. In contrast, other abdominal viscera and peritoneal surfaces remained only normally moist as nearly as could be judged by inspection.

The extrahepatic liver lymphatics were engorged with clear lymph (though of bluish tint from the Evans blue). However, owing to the diverse size and distribution of the lymph channels it was not always possible to isolate and cannulate them. In one animal, where ascites formation was in active progress, hepatic lymphatic engorgement was not present. The lack of lymphatic engorgement in this instance was not explained.

The ascitic fluid of all of the animals was pink due to suspended red blood cells; however, no gross hemolysis was noted after centrifugation. Specimens of hepatic lymph were clear. The average red cell count of the ascitic fluid of the seven animals was 50,000 per cubic millimeter. Red cell counts were somewhat less for hepatic lymph. On the other hand, capsular liver fluid was always pink and generally contained 100,000 red cells per cubic millimeter. This fluid clotted rapidly and always exhibited slight hemolysis. The capsular fluid also contained Evans blue dye. The dye usually appeared in this fluid within ten minutes following injection into the portal vein; appearance of the dye in the hepatic lymph was frequently sooner.

Certain difficulties were encountered in the collection of these fluids. While great care was taken to avoid trauma to the congested liver, some retraction of the organ was necessary to place the collecting sheets under the left lobe and to cannulate the lymphatics. These procedures frequently provoked minimal surface petechial bleeding, controlled by the application of fibrin foam to the bleeding points. To avoid contamination of the specimens with blood, initial collections of capsular liver fluid were made before cannulation of the lymphatics was attempted.

The results of the protein determinations of plasma, liver lymph, capsular liver fluid, and ascitic fluid are shown in Table I. Liver lymph specimens were not obtained in Experiments 4 and 7 because of inability to cannulate the vessels. The samples of ascitic fluid were taken before laparotomy.

TABLE I. TOTAL AND FRACTIONAL PROTEIN VALUES OF PLASMA, HEPATIC LYMPH, CAPSULAR LIVER FLUID AND ASCITIC FLUID IN SEVEN ASCITIC DOG PREPARATIONS. PROTEIN VALUES ARE EXPRESSED IN GRAMS PER 100 ML.

EXPERIMENT	PLASMA			LIVER LYMPH			CAPSULAR LIVER FLUID			ASCITIC FLUID			REMARKS
	TOTAL	ALB.	GLOB.	TOTAL	ALB.	GLOB.	TOTAL	ALB.	GLOB.	TOTAL	ALB.	GLOB.	
1	3.4	1.7	1.7	3.3	1.8	1.5	3.7	2.5	1.2	2.6	1.3	1.3	Marked hemol. liver fluid
2	5.7	2.9	2.8	4.9	2.4	2.5	5.3	2.7	2.6	3.7	1.8	1.9	
3	4.2	2.2	2.0	3.9	2.2	1.7	3.6	2.1	1.5	2.6	1.4	1.2	
4	3.8	2.2	1.6	—	—	—	3.2	2.0	1.2	2.7	1.5	1.2	Lymphatic cannulation impossible
5	3.6	2.4	1.2	3.4	2.0	1.4	3.4	2.1	1.3	2.0	1.3	0.7	Clotting of liver fluid
6	4.9	2.4	2.5	4.5	2.0	2.5	4.5	2.6	1.9	3.3	1.8	1.5	
7	3.8	2.7	1.1	—	—	—	4.0	3.0	1.0	2.7	1.8	0.9	Liv. lymph vessels not cannulated

These data indicate that the protein values of plasma, hepatic lymph, and capsular liver fluid were within similar range; those of the plasma were usually somewhat higher. The parallelism of protein content was especially

striking in the albumin fractions of the fluid from these sources. Ranges of the globulin fractions were often wider between plasma and capsular liver fluid. In Experiments 1 and 7 the total proteins and albumin fractions of the capsular fluid slightly exceeded those of the plasma. The cause of this discrepancy is not clear. Perhaps hemolysis in the capsular fluid was sufficient to increase the apparent colorimetric end point.[4] However, the general similarity of the liver lymph and capsular fluid to plasma is evident.

The protein content of the ascitic fluid in these experiments averaged about 60 per cent of that of the plasma, hepatic lymph, and capsular liver fluid (Table I). Compared with the fluids from the other sources, both the albumin and globulin fractions were proportionately reduced in the ascitic pool.

DISCUSSION

It is well established that chronic hepatic congestion in dogs may provoke voluminous ascites, particularly when the animals receive additional salt.[2, 6, 10, 12, 16] Therefore, examination of the liver and other abdominal viscera of ascitic dogs at laparotomy appeared to offer a logical approach to a study of the site of origin of the ascites. The obvious extrusion of fluid from the capsular surface of the congested liver was the most striking feature of our observations. In fact, if the liver surface were gently dried by blotting, large droplets of clear fluid immediately re-formed and coursed in streams from the liver to the peritoneal cavity. These livers were enlarged, intensely turgid, and congested. The similarity of the capsular liver fluid to hepatic lymph and to plasma is evident from the tabulation. McCarrell and associates[8] have pointed out the close identity of liver lymph and plasma; they remarked that this could only mean that the liver cells are bathed in blood plasma. That the hepatic vasculature is normally very permeable to plasma proteins, which then appear in large quantities in the lymph, was first suggested by Starling[17] and has been amply confirmed by other workers[3, 7, 8, 9] and by ourselves. It appears probable that the capsular fluid, which is constantly shed during hepatic congestion, may represent a frank extrusion of hepatic lymph. Furthermore, the abundance of the capsular fluid, compared with the absence of excessive fluid on the peritoneal surfaces elsewhere, suggests that the bulk of the ascitic fluid may derive directly from the liver.

The contention that ascitic fluid may originate from the engorged liver is not new. Bolton and Barnard[12] observed that ascites occurred in experimental animals only when venous congestion involved the liver. They suggested that liver lymph escaped through the capsule into the peritoneal cavity. Volwiler and co-workers[16] postulated that additional fluid extravasates from the hilar lymphatics during congestion of the organ. Freeman[14] mentioned an interesting experiment in dogs where the liver was transposed to the right thoracic cavity by incising the right diaphragm and then rejoining it below the liver. Subsequent stricture of the thoracic inferior vena cava produced an hepatic effusion entirely contained in the right chest. The composite trends of evidence suggest that the ascites in our experiments largely originates as a direct extravasation of hepatic lymph.[1, 13]

The mechanism of the passage of a lymphlike fluid through the exterior of the liver is not clear. Anatomical studies indicate that hepatic lymph collects in potential spaces (the spaces of Disse) between the cells and the sinusoids and from there drains into minute channels at the periphery of the lobules. These channels, in turn, connect with others as part of a vast lymphatic tree which drains the entire organ through a number of vessels at the hilum. It appears probable that under conditions of excessive lymph production (as from engorgement) the lymphatic system may be overwhelmed; surface extrusion of lymph then offers a less resistant avenue of escape at the subcapsular extremities of the lymphatics.[12] Winter[11] has shown that the hepatic lymph spaces become engorged when congestion or hepatic inflammation occurs. Intense pericapillary edema of the tissue then ensues.

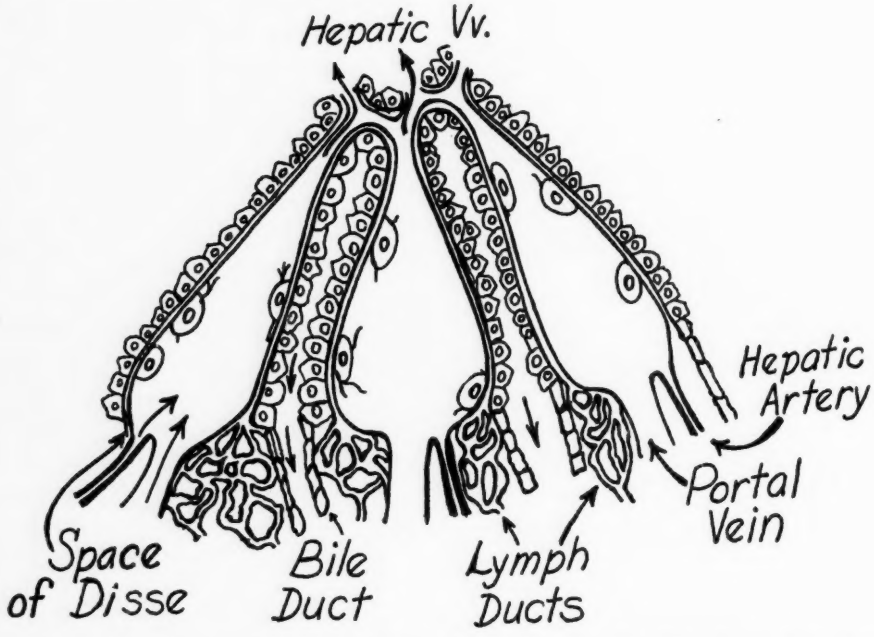

Fig. 1.—Diagrammatic sketch of a liver lobule, showing the disposition of the hepatic cells in relation to the sinusoids. Lymphatic spaces are represented between the cells and the sinusoidal walls with drainage toward the portal areas in parallel with biliary canaliculi.

It has been repeatedly observed in similar ascitic dog preparations that the protein content of ascitic fluid is significantly less than the plasma protein value, a fact which is also evident in our results. If the ascitic fluid is in large measure the product of hepatic lymph extrusion, the question arises as to the mechanism of the diminution, or dilution, of the protein content of the ascitic pool. Two possibilities may be considered. First, one might presume that a selective resorption of protein from the peritoneal fluid takes place, leaving a fluid of reduced protein concentration. We know of no experimental evidence to support this presumption. The second possibility entails the question of a simultaneous movement of water and electrolyte from other

peritoneal surfaces, provoking an increase in the volume of the ascites and a corresponding dilution of protein. This explanation is supported by the work of Courtice and associates.[15] They found that whole blood introduced into the peritoneal cavity of experimental animals was greatly diluted before resorption of the blood occurred. In our observations, therefore, the proteinous hepatic effusion may have competed osmotically for subserosal interstitial fluid, resulting in the passage of water and electrolyte (and perhaps some protein) into the ascitic bulk. The small increment in portal venous pressure may have aided such a tendency to transudation. On the other hand, the minimal elevation of portal pressure observed in these experiments probably had only a minor effect on initiating ascites formation. Experimental portal obstruction rarely induces ascites unless plasmapheresis has been carried out as well. Under such conditions the resultant ascites is serous in quality, low in protein content, and usually scant. It is quite unlike the voluminous, proteinous ascites of hepatic congestion.

SUMMARY

Massive ascites was produced in dogs by constriction of the thoracic inferior vena cava and the supplementary administration of salt.

A voluminous escape of fluid from the capsular surface of the congested liver was observed at laparotomy in these ascitic dog preparations. The protein content of the capsular liver fluid was similar to that of plasma and hepatic lymph. The other peritoneal surfaces showed no conspicuous transudation.

It is postulated that in these ascitic dog preparations the bulk of the protein and water of the ascitic fluid derives directly from the liver as a lymphlike transudate. The water and electrolyte content of the ascitic pool may be supplemented by transudation from the peritoneal surfaces.

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COMPARATIVE VALUE OF THE BASAL METABOLIC RATE, CHEMICAL PROTEIN-BOUND IODINE, AND RADIOACTIVE IODINE EXCRETION OR UPTAKE IN THE DIAGNOSIS OF BORDERLINE HYPERTHYROIDISM WHEN USED INDIVIDUALLY OR IN COMBINATION

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MANY reports have appeared attempting to compare the usefulness of various measures in the diagnosis of hyperthyroidism. Typically the comparisons among tests consist of listings of the percentage overlapping of hyperthyroid and euthyroid groups. Heretofore, few analyses have attempted to take into account quantitatively the interrelationships among the various measurements, and none has attempted to combine the information obtained from the individual measurements. The three most widely discussed measurements are the basal metabolic rate, chemical protein-bound iodine, and radioactive iodine uptake (or excretion) in twenty-four hours. An evaluation of the comparative effectiveness of these tests and of a combination of these tests in distinguishing between borderline euthyroid and borderline hyperthyroid patients—the two most closely contiguous groups in a spectrum from obvious normality to obvious abnormality—is the objective of the present analysis.

METHODS AND MATERIALS

Case Material.—The raw data are available in the extensive report published by Skanse in 1949.[1] The *euthyroid* group consists of 42 cases selected, because complete data were available, from a larger group of 84 patients having diseases that may simulate thyrotoxicosis. Hyperthyroidism was considered initially in three-fourths of them. All were adjudged euthyroid after study and extended follow-up. Cases with overt congestive heart failure, Parkinson's disease, or alcoholism were omitted. Nineteen patients had nontoxic goiters, seven thyroid carcinoma, three exophthalmos without Graves' disease, and two chronic thyroiditis. Fifteen had an anxiety neurosis with or without another complicating disease.

The *hyperthyroid* group consists of 42 cases chosen at random from a sample of 53 who were selected in turn, because complete data were available, from a larger group of 81 patients in whom the clinical diagnosis of thyrotoxicosis was not established at the time of testing, but whose subsequent studies, clinical course, and response to treatment seemed to establish the diagnosis with reasonable certainty. These patients were, in general, older than those with typical hyperthyroidism, and typical symptoms were less frequent. One-third had complicating diseases. Four of the complications were cardiopulmonary. Details concerning these cases may be found in Tables 21, 22, 32, 33, 36, 38, 40, and 41 of the published report. In each of the two groups the ratio of men to women was one to three.

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Variables.—The primary measurements to be evaluated are the basal metabolic rate (B.M.R.), chemical protein-bound iodine (PBI), and percentage urinary radioactive iodine excretion in 24 hours (RI). The latter is, in effect, the complement of the uptake by the thyroid gland, but not as satisfactory a measure for ordinary purposes, because urine collection must be accurate and renal function normal. The correlation between the 24- and 48-hour I^{131} excretion in these patients was 0.98. The correlation between the I^{131} uptake directly measured and that indirectly calculated from the 48-hour urinary excretion values of 136 patients was found by Skanse to be 0.97. The uptake and excretion of I^{131} are thus interchangeable for purposes of the present analysis. Details of methodology are to be found in reference 1.

In addition to these three measures, total cholesterol values were available for 24 euthyroid and 40 hyperthyroid individuals. Data were also available for three descriptive variables—age, pulse, and pulse pressure.

Analysis.—Our objectives were (a) to compare the effectiveness with which each test individually differentiates the two groups, (b) to combine the tests linearly in optimal fashion and to evaluate the independent contribution of each test to this combination, and (c) to determine how much of the observed effectiveness of each test is accounted for by factors underlying the independently significant tests. The statistical procedures utilized in accomplishing these objectives have been described explicitly in this connection in reference 2, and are discussed in general in reference 3.

For proper evaluation of the discriminative effectiveness of a test, one must give consideration to every case in each group, not just those at the extremes of the two distributions. Over-all separation of the two groups should be measured as well as the scatter of cases and incidence of overlapping at the extremes. A satisfactory measure of the relative effectiveness of a test will thus take into account the average differences between the two groups as well as scatter of cases within each group. Such a measure is the *discrimination ratio,* which is that fraction of the total variation of both groups together that is attributable to average differences between the groups. It is utilized in the present analysis.

TABLE I. SUMMARY DATA FOR EUTHYROID AND HYPERTHYROID GROUPS

	EUTHYROID					HYPERTHYROID				
VARIABLE	NO. OF CASES	MEAN	SD	RANGE	PER CENT OVER-LAP*	NO. OF CASES	MEAN	SD	RANGE	PER CENT OVER-LAP*
Age, years	42	44	15	17-72	--	42	48	15	23-76	--
Pulse, beats/min.	37	85	17	60-132	27	42	92	15	70-130	50
Pulse pressure, mm. Hg	35	54	16	30-90	40	40	59	19	30-120	50
Cholesterol, mg. %	24	208	65	98-390	46	40	178	47	78-335	33
B.M.R., %	42	10	16	-16 to +49	41	42	21	14	-6 to +63	31
PBI, μg %	42	5.3	1.5	3.1-11.7	4.8	42	10.2	2.9	4.7-17.9	16.7
RI, %	42	60	11	43-87	2.4	42	28	14	8-64	9.5
Combined score, units	42	+26	12	+6 to +54	0	42	-26	18	-70 to +5	7.1

*Percentage overlapping mid-point between the means of the two groups.

TABLE II. COMPARATIVE DISCRIMINATIVE EFFECTIVENESS OF SEPARATE TESTS AND OF THE COMBINED SCORE

VARIABLE	DISCRIMINATION RATIO (×100)	
	OBSERVED (PER CENT)	RELATIVE
Combined Score	74.6	1.16
RI	64.3	1.00
PBI	53.3	0.83
B. M. R.	12.1	0.19
Cholesterol	6.5	0.10

Fig.

RESULTS AND INTERPRETATIONS

The Tests Considered Separately.—The frequency of occurrence of various values of each diagnostic measure, as well as certain descriptive variables, is portrayed in Fig. 1. For each measure the class intervals along the abscissa are one-sixteenth of the range of values observed for all 84 cases. The characteristics of each distribution are summarized in Table I.

The relative effectiveness of each test in discriminating between the two groups is listed in Table II. The most effective individual test is the RI, for which the difference between means is 64.3 per cent of the total variation observed with the test. The PBI is slightly less effective. The B.M.R. and

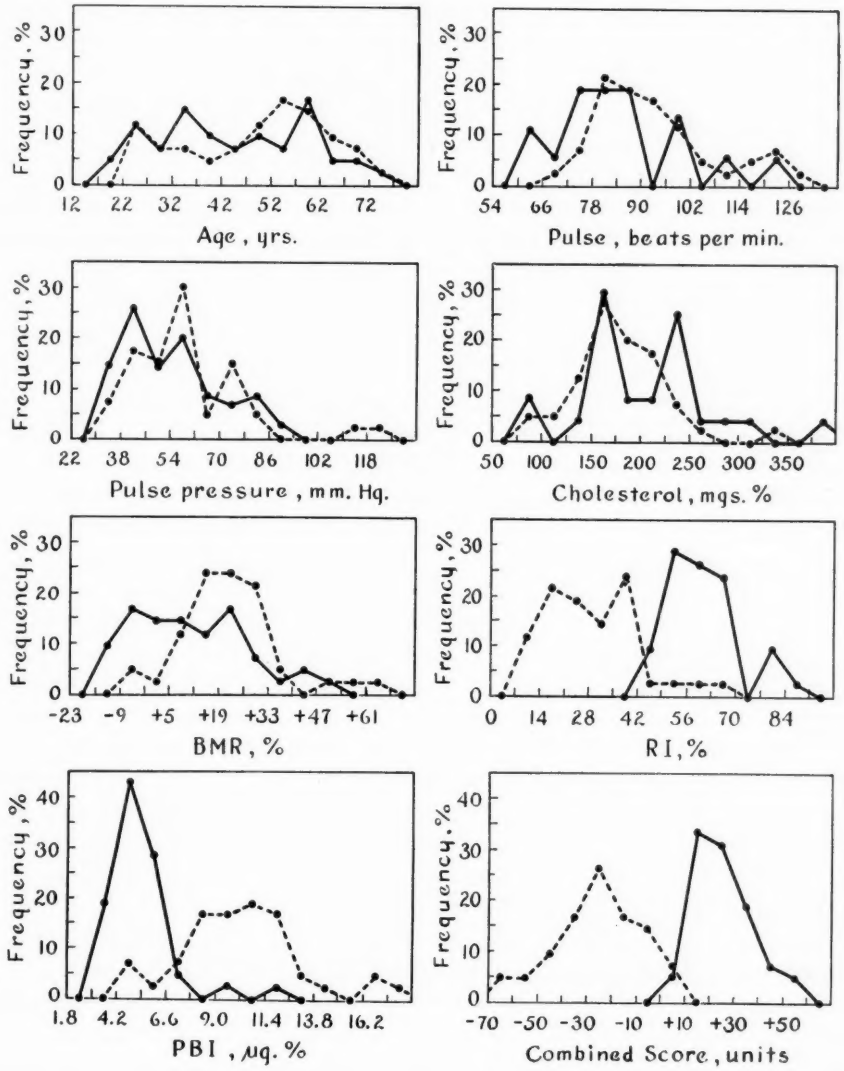

Fig. 1.—Frequency of occurrence of various values of each measure, by group. Solid line, euthyroid. Broken line, hyperthyroid.

cholesterol are only 19 and 10 per cent as effective, respectively. This order of effectiveness of the tests is reflected also in the percentage of cases overlapping the mid-point between the means as shown in Table I.

The Tests Considered Together.—The three most important tests (RI, PBI, and B.M.R.), for which complete data were available, were combined linearly in optimal fashion by deriving weighting factors which represent the independent contributions of the tests and which maximize the discrimination ratio of the combined score. The weighting factor obtained for the RI test was 6.1 times its standard error, for the PBI 4.9 times its standard error, and for the B.M.R. 1.2 times its standard error, the latter being insignificant. The discrimination ratio of the weighted combination of all three tests was 75.1 per cent, a value that is insignificantly different from that of 74.6 per cent obtained for the combination of the RI and PBI alone. The contribution of the RI to the combination was 26 per cent greater than that of the PBI. The formula for calculating the combined score is RI–4PBI–13.

The B.M.R. contributed little information beyond that already represented in the RI and PBI tests. From an analysis of covariance one finds that 47 per cent of the observed discriminative effectiveness of the B.M.R. was contributed by factors which it has in common with the PBI test, and 42 per cent by factors held in common with the RI test.

The RI and PBI also have much in common. For this reason, combining these two optimally only raises the discrimination ratio from 64.3 per cent observed with RI alone to 74.6 per cent. However, this increase of 10.3 per cent is highly significant. Factors accounting for the independent significance of both tests operate to produce the discriminative effectiveness *observed* with each test. Thus in an analysis of covariance we find that 46 per cent of the discrimination ratio observed for the PBI alone was due to factors held in common with the RI test. Likewise 29 per cent of the discrimination ratio observed for the RI alone was due to factors held in common with the PBI test.

COMMENT

The comparisons of the present report represent a stringent test of the diagnostic effectiveness of the measures considered, since the two groups being compared are so closely apposed along the spectrum from euthyroidism to hyperthyroidism. These are also the cases where the tests are most needed.

Clearly the B.M.R. is of small value in these patients. The PBI and RI are both of great value, the latter somewhat more than the former. As one might have expected, a weighted combination of these two measurements is better, although not greatly so, than either one alone.

With these groups the B.M.R. is at a disadvantage because of its nonspecificity. With any groups however, one would expect to find the observed effectiveness of the B.M.R. accounted for by factors underlying the independent significance of the PBI and RI, as was true in the present instance.

The PBI and RI have many factors in common fundamentally, and it would be erroneous to think of the two as independent, one measuring iodine

assimilation and the other hormone production. Both, however, are needed for maximum diagnostic effectiveness, and a weighted combination of the two avoids overemphasis or underemphasis of either. It is of interest that a larger fraction of the observed discriminative effectiveness of the PBI is due to factors accounting for the independent significance of the RI than vice versa.

Sooner or later anyone who deals with thyroid disease over an extended period feels the need for the PBI. However, it is time consuming, expensive, and difficult, and therefore not readily available. Fortunately, factors determining its independent value influence the observed effectiveness of the radioactive iodine studies, and the RI alone is nearly nine-tenths as effective as the combination.

SUMMARY

A comparison has been made of the relative value of the basal metabolic rate (B.M.R.), chemical protein-bound iodine (PBI), and radioactive iodine excretion in twenty-four hours (RI) for purposes of distinguishing between borderline euthyroid and borderline hyperthyroid patients.

Individually considered, the RI was most effective. The PBI was approximately four-fifths as effective and the B.M.R. one-fifth as effective.

A weighted combination of the tests was slightly more effective than the RI. The B.M.R. contributed little to this combination. Eighty-nine per cent of its observed effectiveness was due to factors it has in common with the PBI and RI.

The PBI and RI are also mutually interdependent. Forty-six per cent of the observed effectiveness of the PBI was contributed by factors held in common with the RI. Similarly, 29 per cent of the observed effectiveness of the RI was contributed by factors held in common with the PBI.

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THE HEMOLYTIC EFFECT OF PRIMAQUINE

VII. Biochemical Studies of Drug-Sensitive Erythrocytes

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ERYTHROCYTES from certain individuals possess an intrinsic defect which makes them unusually sensitive to the hemolytic action of primaquine, phenylhydrazine, sulfanilamide, acetanilid, and certain other drugs.[1, 2]

In an earlier study, primaquine-sensitive and primaquine-nonsensitive red cells were compared using hematologic techniques employed to detect previously described intrinsic red cell defects. Examination of fragility to a variety of agents, morphology, sensitivity to acid hemolysis, antigenic pattern, and hemoglobin types failed to reveal any difference between sensitive and nonsensitive erythrocytes. It was observed, however, that in vivo Heinz body formation was more marked in sensitive than in nonsensitive erythrocytes when primaquine was administered.[3] It was also shown that only the older members of red cell populations were destroyed when primaquine was given.[4]

Red cells contain many complex enzyme systems and carry on a number of active metabolic processes. Older erythrocytes contain lower concentrations of many of the red cell enzymes and coenzymes than do young cells.[5, 6] This suggested that sensitive erythrocytes might be deficient in one of these enzymes or coenzymes, the deficiency becoming sufficiently great as they aged to permit hemolysis by otherwise harmless doses of drugs.

A number of different experimental approaches were used to determine which, if any, of the red cell enzyme systems might be involved in unusual sensitivity of red cells to hemolysis. In the case of glycolytic enzymes and catalase the activity of the enzyme was measured; in the case of the sulfhydryl compounds direct chemical means of measurement were used; in all cases the effect of inhibition of the enzyme system under study on Heinz body formation was investigated. Heinz bodies are refractile particles believed to be a manifestation of red cell damage.[7] We had previously shown that there is a close relationship between their formation and primaquine-induced hemolysis: (1) when primaquine is administered, they appear in large numbers in erythrocytes of sensitive individuals but are only rarely seen in the erythrocytes of nonsensitive individuals;[3] (2) reticulocytes, which are refractory to Heinz body formation,[8, 9] are also resistant to hemolysis[4]; (3) red cells sensitive to hemol-

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ysis are also unusually sensitive to in vitro Heinz body formation by acetylphenylhydrazine.[10] We, therefore, consider Heinz body formation as the first stage in the hemolytic process, a stage which can be observed in vitro. Thus, a finding that inhibition of a certain red cell enzyme system caused nonsensitive cells to form Heinz bodies like sensitive cells would suggest that sensitivity to hemolysis might be due to a deficiency in that enzyme system. Conversely, if inhibition of an enzyme failed to influence Heinz body formation, drug sensitivity could not be entirely due to a deficiency of the enzyme system concerned.

The reason for the selection of each enzyme system studied and the methods of investigation employed will be described individually. The selection of volunteers from whom blood samples were obtained has been described previously.[10]

A. GLYCOLYSIS

1. *Rationale:* Glycolysis appears to be the red cell's main source of energy. In the red cell, as in other tissues, this process results in the reduction of diphosphopyridine nucleotide (DPN) and in the formation of adenosine triphosphate (ATP) by phosphorylation of adenosine diphosphate.[11, 12] A number of other red cell defects have already been shown to be due to abnormalities of glycolytic or associated enzyme systems. It has been suggested that the intrinsic defect of congenital spherocytosis is of this type.[13] There is also evidence that the "storage lesion" of erythrocytes is due, at least in part, to a deficiency of ATP and 2, 3-diphosphoglyceric acid.[14] Congenital methemoglobinemia has been shown to be due to a deficiency of coenzyme factor I (diaphorase I), which, together with reduced DPN, normally reduces methemoglobin.[15] It appeared reasonable, therefore, that an abnormality of glycolysis might also be responsible for the defect of primaquine-sensitive erythrocytes.

2. *Methods.*

a. P^{32} uptake.—During glycolysis, inorganic phosphate enters the red cell at a rate approximating glucose utilization.[11] Thus, the P^{32} uptake may be used as an easily measured index of the rate of glycolysis. P^{32}-labeled phosphate uptake of red cells was measured by a modification of the method of Feinstein and associates,[16] employing a scintillation-type counter instead of a Geiger-Müller tube.

Heparinized whole blood was centrifuged, the plasma was removed, and the erythrocytes resuspended with sufficient physiologic saline solution to restore approximately the original volume. One milliliter of saline solution containing 0.5 to 2.5 μc of carrier-free P^{32}* as phosphate was added to 4 ml. of the red cell suspension in a calibrated 12 ml. centrifuge tube. The mixture was incubated for two hours with gentle agitation every ten minutes. The suspension was then chilled in an ice bath for five minutes, centrifuged, the supernatant removed, and the cells washed two times in physiologic saline solution. After the second washing the volume of the packed cells was recorded and sufficient saponin solution was added to make a total volume of 10 ml. After hemolysis of the red cells was complete, duplicate 4 ml. aliquots were pipetted into glass test tubes and were counted in a scintillation-type counter. The percentage of the P^{32} taken up per milliliter of red cells was recorded.

*The P^{32} used in this study was obtained from the Abbott Laboratories under an authorization from the Isotopes Division, United States Atomic Energy Commission, Oak Ridge, Tenn.

A large loss of counts by internal absorption in the solution, by the test tube, and by the tube holder occurs when this modification of the method of Feinstein and associates[16] is used. However, the larger fraction of the sample counted adequately compensates for this loss. Results were highly reproducible, and considerable technical simplification resulted.

b. Effect of inhibitors of glycolysis on Heinz body formation.—The effect of two inhibitors of glycolysis, fluoride and iodoacetate, on Heinz body formation was studied. The oxidation of glyceraldehyde 3-phosphate to glycerate 1, 3-diphosphate with formation of reduced DPN is blocked by iodoacetate.[12] Fluoride inhibits the conversion of glycerate 2-phosphate to pyruvate 2-phosphate.[12] Concentrations of 37.2 mg. per cent (.002 M) iodoacetic acid or of 84 mg. per cent (.02 M) sodium fluoride are sufficient to inhibit glycolysis completely.[17]

The effect of fluoride and iodoacetate on Heinz body formation was studied in erythrocytes incubated for four hours with buffered 100 mg. per cent acetylphenylhydrazine solution containing the inhibitor. The standard procedure used to produce and evaluate Heinz body formation has been described in detail previously.[10]

3. *Results:*

a. P^{32} uptake.—The phosphorus uptake of the erythrocytes of the five sensitive and seven nonsensitive individuals examined did not differ significantly. Primaquine, aniline, pamaquine, phenylhydrazine, iodoacetate, and fluoride all inhibited phosphate uptake to the same extent in sensitive and nonsensitive cells.

b. Effect of inhibition of glycolysis on Heinz body formation.—Two-hundredths molar sodium fluoride failed to influence Heinz body formation. Iodoacetic acid (buffered to pH 7.6), on the other hand, caused a distinct increase in Heinz body formation in concentrations as low as 4.65 mg. per cent (.00025 M). At a concentration of 37.2 mg. per cent (.002 M) the increase was very great, Heinz body formation in nonsensitive cells being fully as great as is ordinarily observed in sensitive cells (Table II). This was true in a lactate as well as in a glucose substrate. In the absence of acetylphenylhydrazine, iodoacetate failed to cause the formation of any Heinz bodies, either in sensitive or in nonsensitive erythrocytes (Table II).

4. *Conclusion:* These studies of the utilization of phosphate by sensitive erythrocytes indicate that glycolysis proceeds at a normal rate in these cells under physiologic conditions, and that the glycolytic system of sensitive cells is no more susceptible to inhibition than that of nonsensitive cells. The fact that inhibition of glycolysis by fluoride failed to influence Heinz body formation strongly suggests that abnormalities of the glycolytic mechanisms of drug-sensitive erythrocytes are not responsible for their unusual behavior when potentially hemolytic drugs are administered.

The observation that small quantities iodoacetate greatly enhanced Heinz body formation may, therefore, be interpreted as an effect on some other enzyme system. The fact that lactate did not abolish the effect of iodoacetate on Heinz body formation (as it does the effect of iodoacetate on methemoglobin reduction[15]) is consistent with this view. The inhibitory action of iodoacetate is known to extend to many sulfhydryl compounds.[18, 19]

B. CATALASE ACTIVITY

1. *Rationale:* Catalase is a hematin enzyme which catalyzes the breakdown of hydrogen peroxide. Its concentration in the red blood cell is second

only to that in the liver.[11] In spite of this abundance of catalase in erythrocytes, the function of the enzyme in erythrocytes is not entirely clear, although it has been postulated that it protects hemoglobin from destruction by peroxide. Ponder[5] has suggested that the gradual depletion of catalase in red cells might be the factor which ultimately leads to senescence and death of normal cells in the circulation.

It has been proposed that ascorbic acid, which forms Heinz bodies in vitro,[10] undergoes coupled oxidation with hemoglobin. A hemoglobin-peroxide intermediary is believed to be formed and subsequently to break down irreversibly to choleglobin.[20] It appeared possible that hemolytic Heinz body-forming compounds might similarly cause formation of a hemoglobin-peroxide compound with the subsequent formation of choleglobin. If this were so, a deficiency of catalase could easily account for the unique susceptibility of sensitive cells to the action of these substances. Jasinski and Müller[21] have suggested that phenacetin sensitivity is due to iron deficiency resulting in catalase deficiency, but have presented no convincing evidence for this view.

2. *Methods:* Catalase activity was measured by the permanganate titration method of v. Euler and associates.[22] The effect of sodium cyanide on Heinz body formation was determined by incorporating buffered sodium cyanide into the buffered acetylphenylhydrazine solution in the standard method we have described for evaluating in vitro Heinz body formation.[10]

3. *Results:* Measurement of the catalase activity of erythrocytes from seven sensitive and five nonsensitive individuals revealed no significant differences. The incubation of the hemolysate with as little as 1.0 mg. per cent phenylhydrazine hydrochloride or 3 mg. per cent acetylphenylhydrazine for one-half hour significantly inhibited catalase activity. However, there was no difference between the degree of inhibition of catalase from sensitive and nonsensitive erythrocytes.

Inhibition of catalase by 1 mg. per cent buffered sodium cyanide failed to influence Heinz body formation.

4. *Conclusion:* Sensitive cells are not catalase deficient. Inhibition of catalase does not appear to make red cells more sensitive to the injurious effect of hemolytic agents. Catalase from sensitive cells is not unusually susceptible to inhibition by hemolytic compounds.

C. CARBONIC ANHYDRASE

1. *Rationale:* Carbonic anhydrase is a red cell enzyme which is known to be inhibited by one of the hemolytic compounds under study, sulfanilamide.[23] Acetazoleamide (Diamox) in concentrations of 0.04 mg. per cent (1.8×10^{-6} M) completely inhibits the carbonic anhydrase activity of dog red blood cells[24] in the in vitro system described by Maren and associates.[24a]

2. *Methods:* Acetazoleamide (Diamox*) was added to the acetylphenylhydrazine solution, and Heinz body formation was measured in the manner previously described.[10]

3. *Results:* Acetazoleamide in concentrations of up to 100 mg. per cent (4.5×10^{-3} M) did not influence Heinz body formation by acetylphenylhydrazine.

*Supplied to us through the courtesy of Dr. James Gallagher, Clinical Research Section, American Cyanamid Company, Lederle Laboratories Division, Pearl River, N. Y.

4. *Conclusion:* Carbonic anhydrase deficiency does not appear to play a role in sensitivity to hemolysis.

D. CHOLINESTERASE

1. *Rationale:* Large quantities of true cholinesterase are present in erythrocytes. The greatest concentrations are present in young cells.[25] The function of this enzyme in red cells is not well understood. It is believed that it may control cell membrane permeability[26] and that it may have an influence on carbohydrate metabolism.[27] Cholinesterase is inhibited by a variety of substances, including physostigmine, the latter almost completely inhibiting red cell cholinesterase activity in concentrations of 0.02 Gm. per cent.[27]

2. *Methods:* Physostigmine sulfate was added to the acetylphenylhydrazine solution and Heinz body formation was measured in the manner previously described.

3. *Results:* Two-hundredths per cent physostigmine sulfate had no effect on Heinz body formation.

4. *Conclusion:* Cholinesterase deficiency does not appear to play a role in sensitivity to hemolysis.

E. NONPROTEIN SULFHYDRYL COMPOUNDS—GLUTATHIONE (GSH)

1. *Rationale:* Glutathione (GSH) is a tripeptide of cysteine, glutamic acid, and glycine, which accounts for over 95 per cent of the reduced nonprotein sulfhydryl compounds in the red cell.[28, 29] Although not itself an enzyme, it is a coenzyme of glyoxalase[30] and, in a tightly bound form, has been shown to be a part of the glyceraldehyde oxidase molecule.[31] It is able to protect against certain injurious agents, including arsenic[32] and x-irradiation,[33] presumably by preserving sulfhydryl enzymes.[34] The fact that the GSH content of red blood cells rises after hemorrhage[29, 35] suggests that young cells contain more GSH than old cells.

We have previously suggested that hemolytic compounds damage the red cell by becoming oxidized and then oxidizing hemoglobin or stroma to form Heinz bodies.[10] GSH is a reducing substance. It seemed possible that it might keep Heinz body-forming substances in the reduced state and thus prevent them from oxidizing hemoglobin or stroma. In this way GSH could protect the red cell against Heinz body formation; conversely, a deficiency of GSH might render the red cell unusually sensitive to Heinz body formation and thus be responsible for unusual sensitivity to hemolysis.

2. *Methods:*

a. Glutathione (GSH) determinations.—

(1) Modification of method of Gruenert and Phillips.—Gruenert and Phillips[36] have described a method of determining GSH which depends on the development of a rose-colored complex of sulfhydryl compounds with sodium nitroprusside and its stabilization with cyanide. We found the following modification simple to perform and able to give reproducible results:

> One milliliter of whole blood was mixed with 2 ml. of 0.5 per cent saponin solution in a test tube. Five milliliters of 3.0 per cent metaphosphoric acid was added during constant agitation of the hemolysate with a stirring rod. Approximately 3 Gm. of sodium chloride was added and the tube was shaken for two minutes. The suspension was filtered through Whatman No. 2 filter paper.

These manipulations were all carried out at room temperature (22° to 30° C.). The filtrate was added to a 25 mm. Coleman Junior spectrophotometer cuvette containing 6 ml. of saturated sodium chloride solution. The mixture was allowed to equilibriate for ten to twenty minutes at 20° C. in a water bath along with a 2 per cent solution of sodium nitroprusside (kept in a dark bottle) and a 328 mg. per cent (.067 M) sodium cyanide-15.9 Gm. per cent (1.5 M) sodium carbonate solution. One milliliter of the sodium nitroprusside solution was layered over the filtrate-saline mixture. One milliliter of the sodium cyanide—sodium carbonate solution was immediately added and the contents of the cuvette simultaneously were mixed with a stirring rod. Within one minute the light absorption at 520 mμ was measured in a Coleman Junior spectrophotometer against a reagent blank containing 6 ml. of saline solution, 2 ml. of 2.0 per cent metaphosphoric acid in saturated saline solution, 1 ml. of the 2.0 per cent sodium nitroprusside, and 1 ml. of the sodium cyanide-sodium carbonate mixture. This blank was stable for several hours. A GSH standard was prepared by dissolving a carefully weighed quantity of glutathione (Schwarz) in 1.875 per cent metaphosphoric acid and saturating this solution with sodium chloride. After filtering, 2.5 ml. aliquots were rapidly frozen and stored at -70° C. Two or more aliquots of the standard were analyzed along with each group of blood filtrates. The GSH content of the blood was calculated according to the linear relationship between light absorption and glutathione concentration (Beer's law), established for this method by Gruenert and Phillips.[36]

(2) Method of Lazarow.—Lazarow and associates[37] have developed a method for determining GSH concentration based on the formation of a compound with an absorption peak at 305 mμ when GSH is mixed with alloxan in a slightly alkaline medium. This method is believed to be highly specific for GSH; sulfhydryl compounds such as systeine, ergothionine, cysteyl glycine, and glutamyl-cysteine give only negligible readings or none at all.[37]

b. Heinz body formation.—Iodoacetic acid and sodium arsenite, buffered to pH 7.5 to 7.6, were added to the acetylphenylhydrazine solution and Heinz body formation measured by the method previously described.[10]

c. Subjects.—GSH determinations were carried out on 93 blood samples from 41 men. Of these men, 8 were primaquine-sensitive and 33 were primaquine-nonsensitive. The sensitivity or nonsensitivity of each of the volunteers was established on the basis of the in vitro test for primaquine sensitivity which we have recently described.[10] In addition, all of the sensitive men and 18 of the nonsensitive men have been given primaquine and their sensitivity or nonsensitivity to hemolysis confirmed in this manner. All 8 of the sensitive men and 26 of the nonsensitive men were American Negroes; 7 of the nonsensitive men were Caucasians. None of the subjects had received any hemolytic drug for at least 10 weeks prior to the time that the GSH determinations were carried out. One of the sensitive men and 22 of the nonsensitive men had never received primaquine or other hemolytic drugs at the time the GSH determinations were carried out.

d. Procedure.—Blood samples drawn from each subject on at least two separate days were analyzed for glutathione and the results of the individual determinations were averaged. All samples were analyzed both by the method of Gruenert and Phillips and by that of Lazarow. In each case duplicate hematocrit determinations were made and the concentration of glutathione in the whole blood divided by the hematocrit to give the concentration in the red cells. This was done because all of the reacting material is present within the red cell.

3. *Results:*

a. *Glutathione (GSH) determinations.*—The results of GSH determinations carried out by the method of Gruenert and Phillips[36] closely paralleled the results obtained by the more specific method of Lazarow (Table I). The concentration of GSH was almost invariably higher in nonsensitive than in

sensitive cells. By employing standard statistical techniques the difference between the mean concentration of GSH in sensitive and nonsepsitive cells could be shown to be highly statistically significant. This was true of the results obtained by either method, but GSH determinations made by the method of Gruenert and Phillips demonstrated the difference between sensitive and nonsensitive cells in the most clear-cut and consistent manner. The GSH concentrations obtained by this method are presented in Fig. 1.

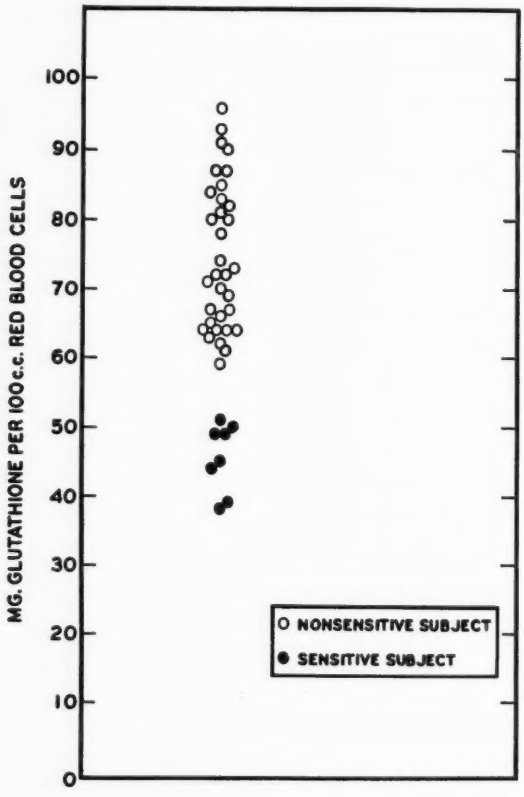

Fig. 1.—Concentration of glutathione in the red blood cells of eight sensitive and thirty-three nonsensitive subjects as measured by a modification of the method of Gruenert and Phillips[36].

b. *Heinz body formation.*—Iodoacetic acid, 37.2 mg. per cent (.002 M) and sodium arsenite, 100 mg. per cent (.0077 M) caused a marked increase in the number of Heinz bodies formed by the action of acetylphenylhydrazine (Table II). A significant increase in the number of Heinz bodies could also be observed with 4.65 mg. per cent (.00025 M) iodoacetic acid and 10 mg. per cent (.00077 M) sodium arsenite. Neither compound caused Heinz body formation in the absence of acetylphenylhydrazine (Table II).

4. *Conclusion:* The red cell glutathione content of primaquine-sensitive erythrocytes is uniformly decreased. Inhibition of sulfhydryl compounds causes nonsensitive cells to behave like sensitive cells.

TABLE I. GLUTATHIONE (GSH) CONCENTRATION IN THE ERYTHROCYTES OF THIRTY-THREE NONSENSITIVE AND EIGHT SENSITIVE SUBJECTS

SUBJECT	RACE	SENSITIVITY TO PRIMAQUINE	MG. GLUTATHIONE (GSH) PER 100 C.C. OF ERYTHROCYTES	
			METHOD OF GRUENERT AND PHILLIPS[36]	METHOD OF LAZAROW[37]
1	Negro	0	64	55
2	Negro	0	87	79
3	Negro	0	91	91
4	Negro	0	71	71
5	Negro	0	87	87
6	Negro	0	93	91
7	Negro	0	81	76
8	Negro	0	96	91
9	Negro	0	84	81
10	Caucasian	0	72	71
11	Negro	0	78	80
12	Negro	0	69	77
13	Negro	0	85	87
14	Negro	0	90	90
15	Negro	0	59	66
16	Caucasian	0	72	71
17	Caucasian	0	67	71
18	Caucasian	0	64	64
19	Caucasian	0	62	61
20	Caucasian	0	73	68
21	Negro	0	80	81
22	Negro	0	83	80
23	Negro	0	61	63
24	Negro	0	80	79
25	Negro	0	67	66
26	Negro	0	70	71
27	Caucasian	0	82	76
28	Negro	0	63	61
29	Negro	0	74	84
30	Negro	0	66	73
31	Negro	0	65	72
32	Negro	0	64	75
33	Negro	0	64	65
Mean GSH concentration in nonsensitive cells			74.67	74.97
Sample standard deviation			10.40	9.51
34	Negro	+	51	57
35	Negro	+	50	54
36	Negro	+	45	51
37	Negro	+	44	51
38	Negro	+	49	51
39	Negro	+	49	54
40	Negro	+	39	46
41	Negro	+	38	43
Mean GSH concentration in sensitive cells			45.63	50.88
Sample standard deviation			4.66	4.20

TABLE II. THE EFFECT OF IODOACETATE AND ARSENITE ON IN VITRO HEINZ BODY FORMATION BY ACETYLPHENYLHYDRAZINE

CONCENTRATION OF ACETYL PHENYL-HYDRAZINE	SULFHYDRYL INHIBITOR	PERCENTAGE OF ERYTHROCYTES FORMING FIVE OR MORE HEINZ BODIES			
		NONSENSITIVE		SENSITIVE	
		A	B	C	D
100 mg. %	None (control)	8	6	52	50
	.0020 M iodoacetate	89	91	99	97
	.0077 M arsenite	98	95	100	99
0	.0020 M iodoacetate	*(No Heinz body formation)*			
	.0077 M arsenite	*(No Heinz body formation)*			

DISCUSSION

In this study we have demonstrated that primaquine-sensitive erythrocytes, although normal in all other respects studied, contain only about 60 per cent of the average normal amount of glutathione (GSH).

There are two possible interpretations of the relationship of this GSH deficiency to drug sensitivity. The GSH deficiency could merely be a secondary effect of some other, as yet undiscovered, abnormality of sensitive red cells; or the deficiency of bound or unbound GSH in these cells could be the cause of their unusual sensitivity to drug administration. Poisoning sulfhydryl groups with iodoacetate or with arsenite caused nonsensitive cells to become as susceptible to in vitro Heinz body formation as only sensitive cells ordinarily are; this is consistent with the concept that a deficiency of bound or unbound GSH is the primary cause of sensitivity to hemolysis.

The means by which GSH may protect red cells against the effect of hemolytic compounds is not entirely clear. A detoxifying role would not be an unusual one for GSH to play, however; it is known to be effective in protecting against arsenic,[32] which in itself may cause a hemolytic anemia,[38] and against ionizing radiations.[33] GSH might protect against hemolysis by keeping the hemolytic compounds in the reduced state, thus preventing them from effectively oxidizing hemoglobin or stroma. We should also mention the possibility that at least a part of the GSH measured in blood filtrates might have originally been split from protein in the precipitating process. If this were the case, sensitive cells could be deficient in a GSH-containing enzyme rather than free GSH itself.

Studies to evaluate further the relationship between drug sensitivity and GSH deficiency are in progress.

SUMMARY

Studies have been carried out to determine the biochemical nature of the intrinsic defect of primaquine-sensitive erythrocytes.

No indication was found that primaquine sensitivity is due to a defect in glycolysis, in catalase activity, in carbonic anhydrase activity, or in cholinesterase activity.

Primaquien-sensitive cells are uniformly glutathione (GSH) deficient.

Iodoacetic acid and sodium arsenite, two sulfhydryl inhibitors, cause nonsensitive erythrocytes to behave like sensitive erythrocytes in vitro with respect to Heinz body formation.

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EFFECT OF A BACTERIAL POLYSACCHARIDE (PIROMEN) IN PREVENTING TISSUE LOSS IN RABBITS DUE TO GANGRENE INDUCED BY FROSTBITE

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THE prevention of gangrene in peripheral vascular diseases has long been an acute problem.[1] In the course of our studies on peripheral vascular disease and gangrene we resorted to the experimental production of gangrene following frostbite.

This manuscript presents the results of this work, which indicate that Piromen* acts as an adjuvant to the physical therapy of rapid thaw in preventing the gangrene and natural amputation which follow freezing of the rabbit ear.

MATERIALS AND METHODS

Several preliminary experiments were carried out in an effort to determine the most advantageous combination of physical therapy with Piromen. The use of gradual thaw alone, or gradual thaw with Piromen, did not prevent gangrene. Rapid thaw therapy and a combination of Piromen with rapid thaw afforded the best protection. The following procedure was adopted as a more formal test:

Sixty-two female New Zealand white rabbits, weighing between 4 and 5 kilograms each, were divided into two equal groups. The left ear of each rabbit was carefully and completely shaved with electric clippers (Oster-surgical head). The right ear was reserved for injections and served as a comparable control. The animals were placed under deep anesthesia (Nembutal) before freezing.

The technique used for the frostbite studies was that of Fuhrman and Crismon.[2] The freezing mixture was maintained at –50° C. and consisted of solid carbon dioxide in a 50:50 mixture of ethylene glycol and water containing 150 ml. of 95 per cent ethyl alcohol per liter. The distal third of the ear was immersed for exactly one minute, care being taken to expose equal amounts of tissue in each case. The ear was then transferred to a solution of water held at +42° C. (rapid thaw) and immersed for two minutes; the animal was then returned to its cage.

Animals which were to receive Piromen or placebo injections were selected at random. Piromen was administered, 1 γ per kilogram (in 0.1 ml.) of body weight intravenously† immediately following rapid thaw therapy, daily thereafter for seven days, then on alternate days for 21 days, week ends excluded. A similar schedule was observed for those animals receiving the placebo (0.1 ml. per kilogram) consisting of sodium-R-lactate with thimerosal (1:10,000).

The animals recovered promptly and were housed in galvanized metal cages. No restraint or protective measures were taken to shield the damaged ears. The rabbits were fed their regular diet while under observation. A 5 per cent solution of sodium fluoresceinate (1 ml. per rabbit) was given intravenously for tissue clearance studies.

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*Piromen is a sterile, nonprotein, and nonanaphylactogenic bacterial polysaccharide. It is prepared as a stable suspension in 1/6 M sodium-R-lactate containing 1:10,000 parts of thimerosal. Piromen is a product of Travenol Laboratories, Inc., a subsidiary of Baxter Laboratories, Inc., Morton Grove, Ill.

†Intravenous injection of 1 γ of Piromen per kilogram of body weight causes an average rise of 0.68° C. in body temperature in the rabbit.

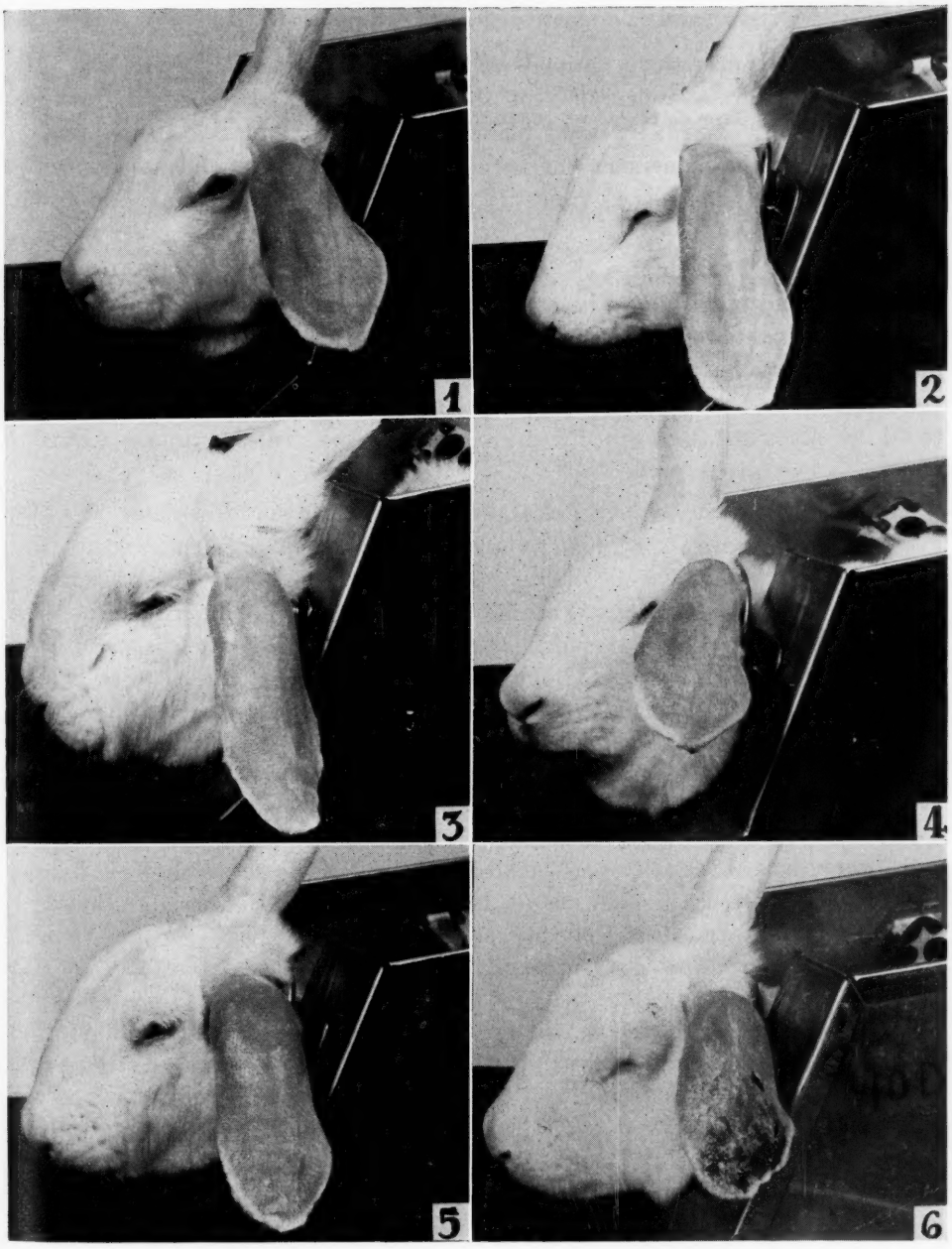

Fig. 1.—One hour after freezing. Rapid thaw and placebo therapy. Ear pendulous from accumulating edema.

Fig. 2.—One hour after freezing. Rapid thaw and Piromen. Ear pendulous as in Fig. 1.

Fig. 3.—Four hours after freezing. Rapid thaw and placebo. Continued accumulation of edema and blood stasis.

Fig. 4.—Four hours after freezing. Rapid thaw and Piromen. Edema diminishing. Animal able to hold ear semierect.

Fig. 5.—Eighteen hours after freezing. Rapid thaw and placebo. Edema persisting and exudative weeping beginning.

Fig. 6.—Ten days after freezing. Rapid thaw and placebo. Ear remains pendulous and covered with thick eschar. Tissue becoming gangrenous.

RESULTS

During the first thirty seconds of exposure to freezing, there was little change in the gross appearance of the rabbit's ear. However, a few seconds thereafter, extremely rapid blanching occurred and the entire area of tissue distal to the immersion line turned snow-white. This did not appear to be a progressive change, i.e., from periphery inward or vice versa, but an all-encompassing effect.

During the two minutes of rapid thawing, the tissue rapidly lost its white appearance and rigidity and became erythematous and flaccid. Moments later, the ear began to swell with edema and became crimson in color. Within one hour the ear was heavy and fully dependent. The entire ear was involved. The venular radicles were markedly distended. Up to this point no differences could be discerned between Piromen- and placebo-treated animals (Figs. 1 and 2).

Four hours later, however, clearance of the edema could be detected in the Piromen-treated animals, while in the placebo-treated rabbits edema and swelling increased (Figs. 3 and 4). Eighteen hours later, there was greater swelling and the process of exudative weeping began (Fig. 5). The Piromen-treated animals continued to show a reduced amount of swelling. At this point some animals were injected with sodium fluoresceinate intravenously, by the technique described by Fuhrman and Crismon[2] and Lange and Boyd.[3] The spread and tissue clearance of this dye were followed under ultraviolet light. The dye appeared in the distal portion of the ear in a few minutes in Piromen-treated rabbits, and within one hour the dye had cleared so completely that the ear showed only a slight fluorescence. During the same period, those ears which had been thawed rapidly, or treated with placebo only, showed fluorescence from the base to the line of immersion only. Hours later, however, fluorescence appeared in the peripheral margins of the distal portions; finally, the dye was concentrated throughout the entire area of tissue damage and gave off a brilliant fluorescence. A considerable amount of dye appeared in the exudate on the surface of the ear. Twenty-four to forty-eight hours after freezing, the dye was still present so that the area of damaged tissue was sharply demarcated from the healthy proximal portions where the dye had cleared.

Several days later the ears of animals given placebo remained dependent and completely covered with eschar from the line of immersion distad. Approximately two weeks after exposure, frank gangrene (Fig. 8) appeared in all parts of the frozen tissue in placebo-treated animals. By the third week, this gangrenous tissue had become dry and mummified. The gangrenous mass separated after the fourth week along the line of immersion by a process of natural amputation (Figs. 9 and 10). Bleeding at the time of separation varied; normally, the immersion line was "sealed over" by connective tissue and epithelial repair so that little, or no, bleeding occurred. Occasionally a rabbit tore this new tissue, and copious bleeding from the many relatively large

and patent vessels took place. By the sixth week, all tissue destined to be lost had separated. (See Table I.)

Animals treated with Piromen, on the other hand, were able to hold their ears naturally erect without a trace of edema. The light coat of eschar which had formed was sloughed off spontaneously and completely, exposing pink, vital tissue (Figs. 6 and 7).

Injections of Piromen or placebo were terminated by the end of the fourth week.

All the ears of animals receiving Piromen in addition to rapid thaw therapy were vital and warm to the touch. Except in a few instances where the tip of

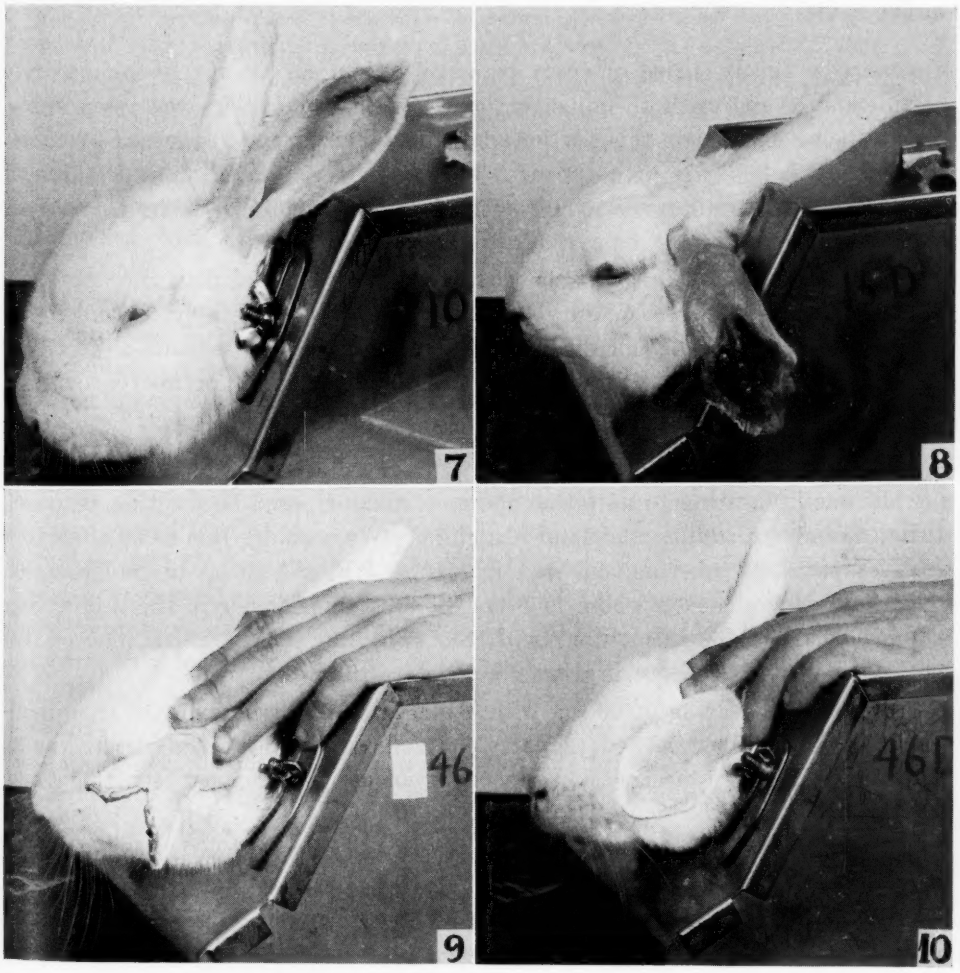

Fig. 7.—Ten days after freezing. Rapid thaw and Piromen. Ear held naturally erect. Eschar sloughed off revealing pink vital tissue. Note regrowth of hair.

Fig. 8.—Fifteen days after freezing. Rapid thaw and placebo. Ear pendulous. Gangrene encompasses entire area of frozen tissue up to line of immersion.

Fig. 9.—Twenty-eight days after freezing. Rapid thaw and placebo. Natural amputation of gangrenous tissue across line of immersion.

Fig. 10.—Twenty-eight days after freezing. Rapid thaw and Piromen. Hair regrowth complete and vascular pattern restored. Ear pliable and warm to the touch.

TABLE I. EFFECTS OF PIROMEN IN PREVENTING TISSUE LOSS DUE TO GANGRENE INDUCED BY FROSTBITE

TREATMENT	NO. OF RABBITS	NO. OF INTACT EARS	PARTIAL LOSS*	TOTAL LOSS
Freezing at −50° C. for 1 min. with rapid thaw at +42° C. for 2 min. Treated with sodium-R-lactate placebo 0.1 ml. per kg.	31	9	1	21†
Freezing at −50° C. for 1 min. with rapid thaw at +42° C. for 2 min. Treated with 1 γ Piromen per kg. I.V. (P-63A)	31	28	3	0†

*Partial loss is construed to be any separation of tissue, no matter how trivial in area.
†The difference between the placebo- and Piromen-treated groups when analyzed by the X^2 method using the Yates correction, proved to be extremely significant.

the ear was slightly curled, the ears were in excellent condition. In the majority of animals the regrowth of hair over the exposed tissue surface was remarkable, growth being heavier on this portion of the ear than on the unexposed proximal portion. This heavy growth appeared to be most accentuated along the line of immersion. Discontinuance of Piromen therapy after four weeks in no way affected recovery.

In a few cases the frozen parts were retained after rapid thaw therapy alone (Table I). However, the surviving tissue was considerably shrunken in length and thickened by fibrosis. The scarified tissue was ischemic and cold to the touch. Re-epithelization was poor and there was little or no regrowth of hair on the damaged tissue.

DISCUSSION

It was interesting to note that the ear, when exposed to freezing temperatures, underwent sudden and total blanching. We consider this to be analogous to the "pinging" phenomenon seen in man. ("Ping" refers to the sensation experienced by a person whose face is exposed to cold, wherein there is a very sudden, sharp pain concomitant with the appearance of a white spot on the cheek which may increase in diameter, i.e., frostbite.[4])

The rapid clearing of edema with a minimum of tissue exudate in animals treated with Piromen was particularly characteristic. This is interpreted as an indication of the restoration of proper physiologic function between the vascular and interstitial beds.

Attempts to explain the histologic processes associated with the healing of the frozen rabbit ears on Piromen therapy will be dealt with at greater length in a subsequent paper. However, it may be pointed out that a number of the known physiologic effects of Piromen may enter into the healing process. Experimentally, striking effects have also been achieved in certain problems related to nerve injury.[5-7] In nerve trauma and other studies of wound healing,[8-10] increased vascularity and macrophage activity, inhibition of glial or scar tissue barriers, and induction of mesenchymal-like tissue growth have been observed in and around the traumatized area.

Similarly Piromen has been definitely shown to stimulate the pituitary-adrenal system, resulting in increased secretion of adrenal corticoids.[11-16] The well-known beneficial effect of adrenal corticoids on capillary permeability or vasomotor tone[17] may be a factor in the healing of the rabbit ears. Further histologic study of the affected parts may throw more light on the mechanisms of the healing process.

Active proliferation of the vascular bed in animals treated with Piromen may account for the rapid clearing of edema, the shortening of the period of exudation, early sloughing of eschar, tissue survival, and hair regrowth. Histologically, it appears that proliferation of capillaries is most marked along the line of immersion, and this is the area, from gross observations, where exceptionally heavy regrowth of hair is most apparent in these animals.

As was stated earlier, the tissues which did survive with rapid thaw therapy alone were shrunken and fibrotic. Specimens removed for histopathologic analyses revealed dramatic fibrotic activity. A heavy deposition of new cartilaginous material was seen to extend from the line of immersion distad on both sides of the endoskeleton of aural cartilage. This was either markedly reduced or, in most cases, entirely absent in animals treated with rapid thaw therapy and Piromen.

In view of the effectiveness of Piromen as an adjuvant to the physical therapy of rapid thaw for frostbite and its possible vascular implications, further studies are being conducted in related vascular pathology such as atherosclerosis, gangrene, and allied disorders.

SUMMARY

The ears of sixty-two rabbits were immersed for one minute in a freezing mixture cooled to –50° C., then rapidly thawed for two minutes in a water bath heated to +42° C. One group of rabbits (31) received 1.0 γ per kilogram of Piromen (a bacterial polysaccharide) per animal intravenously daily for thirty days; placebo injections of sodium-R-lactate (0.1 ml. per kilogram) were given intravenously to animals[31] in a second group.

Of the rabbits given rapid thaw therapy and placebo, 32 per cent suffered no loss of exposed tissue by amputation, but the ears were nonvital in appearance. Animals given rapid thaw therapy and Piromen approached 100 per cent recovery, these ears being in excellent condition.

It appears that Piromen enhanced the effectiveness of rapid warming of rabbit ears exposed to freezing temperatures.

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ISOLATION OF HISTOPLASMA CAPSULATUM FROM FRESH AND DEEP-FROZEN PERIBRONCHIAL LYMPH NODES OF DOGS BY MOUSE INOCULATION

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INTRODUCTION

IN PREVIOUSLY reported studies of naturally acquired histoplasmosis in dogs and cats, peribronchial lymph nodes yielded the highest percentage of positive cultures.[1, 2] However, this tissue frequently contains viable spores of saprophytic fungi and occasionally bacteria which presumably have been inhaled recently by the animal. Most saprophytic fungi and bacteria grow much more rapidly than Histoplasma and, when present in cultures, may obscure or prevent the growth of Histoplasma.

The growth of bacteria from a contaminated inoculum can be prevented by incorporating antibiotics in the culture medium, but a selective culture medium suitable for Histoplasma and unsuitable for saprophytic fungi has not yet been described. Histoplasma was first isolated from the rich and varied microflora of soil by injecting mice with soil suspensions.[3] Mice were sacrificed 4 to 6 weeks after injection and their spleens and livers were cultured for fungi. In this procedure the mouse acts as a host selective for the pathogen. In the present study mice were used similarly for isolating Histoplasma from both fresh and deep-frozen peribronchial lymph nodes of the dog.

MATERIALS AND METHODS

Dogs.—One hundred eleven apparently healthy adult dogs were collected from their owners in Loudoun County, Virginia. These dogs include 111 of the last 117 from a series previously reported.[2] The omissions include 5 young pups and one adult dog which died and was suspected of having rabies. This series of 111, therefore, represents an otherwise unselected consecutive series.

Cultures of Peribronchial Lymph Nodes.—The lymph nodes were carefully dissected from the bifurcation of the trachea and from the main bronchi. Approximately one-half of each node was cultured directly on a slant of Sabouraud's glucose agar. Usually 4 or 5 direct cultures of these nodes were obtained from each dog.

The remaining portions of lymph nodes from each dog were pooled and used for inoculation of mice. Portions of lymph nodes from 92 dogs were homogenized and injected at once into mice. Portions of lymph nodes from 19 dogs were frozen at about −40° C. for 23 to 160 days before homogenization and injection into mice. Frozen tissues were thawed to room temperature before homogenization.

For homogenization the pooled lymph node portion was macerated with sterile scissors and ground manually with a mortar and pestle in a small amount of sterile saline solution. Equally divided portions of 3 to 6 ml. of homogenate obtained from the lymph nodes of each

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dog were injected intraperitoneally into 4 young adult, male, Swiss white mice. The mice were sacrificed 4 to 6 weeks after injection, and cultures of mouse livers and spleens were made on slants of Sabouraud's glucose agar. Homogenates from 2 of the 111 dogs killed all mice 3 to 6 days after injection before adequate cultures for fungi could be obtained.

Direct Cultures of Other Fresh Tissues.—From each dog 20 or more cultures of cervical, mesenteric and other lymph nodes, submaxillary gland, liver, spleen, and adrenal were made on slants of Sabouraud's glucose agar as previously described.[1]

All cultures of dog and mouse tissues were incubated at 30° C. and held for 6 weeks before discarding. All cultures of Histoplasma were identified by the characteristic microscopic morphology of the organism.

RESULTS

1. *Isolation of Histoplasma by Injection of Mice With Fresh Lymph Nodes.*—Histoplasma was isolated from 48 of 92 dogs by injection of homogenates of fresh peribronchial lymph nodes into mice. Histoplasma was isolated from other tissues of 3 additional dogs by direct culture. Contaminants overgrew all direct cultures of the peribronchial lymph nodes from these 3 dogs and all mice receiving homogenates of lymph nodes from 2 of the 3 died 3 to 6 days after injection.

The results of cultures from the 51 infected dogs are presented in Table I. Direct cultures of peribronchial lymph nodes of 12 dogs were positive for Histoplasma, and cultures of mice receiving homogenates from these 12 dogs were also positive. The organism was isolated from mice receiving homogenates from an additional 36 dogs; all direct cultures of peribronchial lymph nodes of 35 of these 36 dogs were contaminated.

All direct cultures of peribronchial lymph nodes of 34 of 41 dogs negative for Histoplasma were contaminated.

TABLE I. ISOLATION OF *Histoplasma capsulatum* FROM 51 DOGS; HOMOGENATES FOR INJECTING MICE PREPARED FROM FRESH PERIBRONCHIAL LYMPH NODES

	CULTURES FOR H. CAPSULATUM		
	PERIBRONCHIAL LYMPH NODES		OTHER TISSUES*
NUMBER OF DOGS	MOUSE INJECTION	DIRECT CULTURES	DIRECT CULTURES
8	+	+	+
4	+	+	0
7	+	0‡	+
29	+	0§	0
3	0†	0‡	+

*Cervical, mesenteric, and other lymph nodes, submaxillary gland, liver, spleen, and adrenal.

†All mice receiving homogenates from 2 dogs died 3 to 6 days after injections.

‡All direct cultures of peribronchial lymph nodes contaminated.

§All direct cultures of peribronchial lymph nodes of 28 dogs contaminated.

The results demonstrate the efficiency of the mouse injection method for isolating Histoplasma from a tissue frequently contaminated with saprophytic fungi and/or bacteria. This method is not indicated for dog tissues which are rarely contaminated. It is apparent that peribronchial lymph nodes are the most frequently infected dog tissue.

The isolation of Histoplasma from an animal population may serve as an index of the infestation of an environment with the organism.[2] An adequate

index could be obtained by culturing only peribronchial lymph nodes of dogs and/or cats. It would be desirable if peribronchial lymph nodes, possibly collected in the field away from laboratory facilities, could be stored and cultured at a later date. Histoplasma has been isolated from experimentally infected mouse tissues stored for several months at –40° C. and repeatedly thawed and refrozen.[4] Portions of peribronchial lymph nodes from a small series of dogs were frozen to determine whether the organism could be isolated from stored naturally infected tissues.

2. *Isolation of Histoplasma by Injection of Mice With Frozen Lymph Nodes.*—Histoplasma was isolated from 12 of 19 dogs by injection of mice with homogenates of peribronchial lymph nodes which had been frozen at –40° C. No additional infected dogs in this group of 19 were found by direct culture.

The results of dog and mouse cultures are shown in Table II. Histoplasma was isolated by direct culture of peribronchial lymph nodes or other tissues from 6 dogs. The organism was isolated from mice receiving peribronchial lymph node homogenates from an additional 6 dogs; all direct cultures of this tissue and other tissues of these 6 dogs were negative for Histoplasma. All direct cultures of peribronchial lymph nodes of 15 of 19 dogs were contaminated.

TABLE II. ISOLATION OF *H. capsulatum* FROM 12 DOGS; HOMOGENATES FOR INJECTING MICE PREPARED FROM FROZEN PERIBRONCHIAL LYMPH NODES

	CULTURES FOR H. CAPSULATUM		
	PERIBRONCHIAL LYMPH NODES		OTHER TISSUES*
NUMBER OF DOGS	MOUSE INJECTION	DIRECT CULTURES	DIRECT CULTURES
2	+	+	+
2	+	+	0
2	+	0†	+
6	+	0†	0

*Cervical, mesenteric, and other lymph nodes, submaxillary gland, liver, spleen, and adrenal.

†All direct cultures of peribronchial lymph nodes contaminated.

Eight of nine isolations from stored peribronchial lymph nodes were obtained from tissues frozen more than 30 days. One isolation was made from tissues stored 160 days. It is apparent that adequate cultures for Histoplasma can be obtained from tissues stored for long periods of time at –40° C.

DISCUSSION

For environmental or clinical pathologic studies, the mouse injection method offers advantages for isolating Histoplasma from potentially contaminated specimens. On the basis of results with naturally infected dog peribronchial lymph nodes, we have adopted the mouse injection method for isolating Histoplasma from certain specimens—sputum, bronchoscopic collections, material from draining sinuses or open ulcers, pulmonary lesions, and peribronchial lymph nodes obtained either at surgery or autopsy. Other specimens such as bone marrow and biopsied lymph nodes are rarely contaminated and may be cultured directly, since it has been demonstrated that positive cultures can be obtained from tissues containing small numbers of viable yeast cells.[5] Homogenates of human peribronchial lymph nodes obtained at autopsy frequently contain bacteria patho-

genic for the mouse. Deaths of mice caused by these organisms can be prevented by homogenizing the tissues in saline solution containing penicillin and streptomycin.

The ability of *H. capsulatum* to survive storage at –40° C. permits use of a laboratory procedure of considerable practical usefulness. When there is delay in transmission of a surgical or post-mortem specimen to a diagnostic laboratory, and in field studies when it is necessary to collect human or animal specimens for shipment to a distant laboratory, this experimental work has demonstrated the feasibility of deep freezing of tissues. While our experience to date with human material has been very limited, we have isolated Histoplasma from one surgical specimen shipped to this laboratory in solid CO_2.

It has been suggested that clinical specimens possibly infected with Histoplasma should be suspended in 2.5 per cent mucin for mouse injections.[6] Very few, probably one, viable yeast cell of Histoplasma is infective for the mouse.[5, 7] The enhancement of infectivity by mucin of small numbers of yeast cells for the mouse, therefore, appears unnecessary.

The number of viable yeast cells of Histoplasma in saline suspension decreases rapidly at room temperature.[5] The number of viable cells in suspension remains constant for several hours in cold saline solution or for longer periods of time in cold buffered cysteine in saline solution.[5] In this laboratory collected specimens are iced immediately and handled in the cold until cultured directly and/or injected into mice.

Homogenizing tissues with a mortar and pestle is a simple and convenient method for handling occasional specimens. For other studies using large numbers of tissues, a Potter-Elevehjem glass homogenizer has been used.[4] Quantitative studies on the number of viable yeast cells of Histoplasma in mouse livers and spleens have demonstrated that this method does not kill yeast cells in these tissues. Peribronchial lymph nodes from 33 dogs were ground with the glass homogenizer. Histoplasma was isolated from mice receiving homogenates from 13 dogs. Positive direct cultures of this and/or other tissues were obtained from 6 of the 13 positive dogs.

SUMMARY

Portions of fresh peribronchial lymph nodes from 92 dogs collected from Loudoun County, Virginia, were homogenized and injected into mice. The mice were sacrificed 4 to 6 weeks after injections, and mouse livers and spleens were cultured for fungi. Direct cultures of peribronchial lymph nodes and 20 or more cultures of other tissues were made on each dog. Histoplasma was isolated from a total of 51 dogs. The organism was isolated from mice receiving peribronchial lymph node homogenates prepared from 48 of the 51 positive dogs. The organism was isolated only by the mouse injection method from 29 dogs. The failure to isolate Histoplasma by direct culture of peribronchial lymph nodes is attributed to the high incidence of contamination of this tissue by saprophytic fungi and occasionally bacteria.

In a subsequent series Histoplasma was isolated from mice receiving homogenates prepared from peribronchial lymph nodes of 12 of 19 dogs. The

lymph nodes had been stored at –40° C. for 23 to 160 days before homogenization and injection into mice.

The application of the mouse injection method for isolating Histoplasma from clinical specimens is discussed.

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HYPOPROTHROMBINEMIA AND HYPOPROCONVERTINEMIA DURING PREGNANCY

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HYPOPROTHROMBINEMIA has been described as a clinical entity by many investigators. It is now recognized that this blood coagulation defect results from a deficiency of the specific enzyme precursor, prothrombin, in the peripheral blood, and that it can arise in a variety of conditions. Dietary deficiency of vitamin K (in experimental animals); impaired vitamin K assimilation in obstructive jaundice, sprue, and celiac disease (and possibly associated with an irradication of K-synthesizing intestinal bacteria during antibiotic therapy); functional liver disease or liver damage (as in deep chloroform anesthesia); and Dicumarol or salicylate poisoning have all been implicated. A coagulation defect in newborn infants has also been attributed to a transitory deficiency of prothrombin, as well as proconvertin. Further, a number of patients with so-called idiopathic hypoprothrombinemia have been described; in few of these cases, however, could the defect be attributed with certainty to a lack of prothrombin alone. Biggs and Douglas[1] have stated: "In five instances the defect was probably a reduction of factor V (Owren, 1947; Frank et al., 1950; de Vries, Matoth, and Shamir, 1951; Stohlman, Harrington, and Moloney, 1951; Brink and Kingsley, 1952). In four instances, factor VII may have been deficient (Giordano, 1943; Crockett, Shotton, Craddock, and Leavell, 1949; Landwehr, Lang, and Alexander, 1950; Alexander, Goldstein, Landwehr, and Cook, 1951). In nine instances the defect was not clearly defined (Rhoads and Fitz-Hugh, 1941; Plum, 1943; Murphy and Clark, 1944; de Marval and Bomchil, 1944; Hauser, 1945; Quick, 1947; Hagen and Watson, 1948; Covey, Cohen, and Papps, 1950; Ferguson, 1950)." Biggs and Douglas, 1953,[1] reported the first undisputed case of an adult who acquired idiopathic prothrombin deficiency.

Proconvertin or factor VII, an accelerator of prothrombin conversion, has been found to be deficient in patients treated with the dicoumarin drugs, in obstructive jaundice and steatorrhea, in a hemorrhagic disease of the newborn infant, and as a congenital coagulation defect. Five such congenital hypoproconvertinemia cases have been reported (Alexander, Goldstein, Landwehr, and Cook[2]; Owren[3]; Lewis, Fresh, and Ferguson[4]; Jenkins[5]).

In this paper is presented the case history of a patient who acquired a definite prothrombin and proconvertin deficiency during the fifth month of pregnancy. Her condition of combined hypoprothrombinemia and hypoproconvertinemia could not be attributed to any of the causes enumerated previously.

From the J. K. and Susie L. Wadley Research Institute and Blood Bank, Dallas, Tex.
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CASE HISTORY

The patient, Mrs. C., was a well-nourished, 31-year-old, white woman, five months pregnant when first seen in the Wadley Clinic in January, 1954. She reported the onset of backache three and one-half days prior to admission. Two days before admission, she also noted a mild, persistent hematuria and a bleeding from the gums, as well as from a small burn on the finger. On the same day as the onset of backache, Mrs. C. had two teeth filled, at which time a local anesthetic was employed and Ag-Hg amalgam fillings inserted. The patient had been taking multivitamin capsules for two months, had taken small doses of Anacin* on two occasions after the onset of backache and hematuria but otherwise had received no medication. She reported the odor of new paint in her home for some time. Dietary intake was good and adequate. A history of drinking eight cups of coffee daily for 6 to 7 years was given. The patient reported general good health all her life. No prior hematuria, melena, nausea, vomiting, or tarry stools were reported. Her past history was noncontributory; no previous operations or injuries were reported. Menstrual history was normal. Mrs. C. had had one prior pregnancy; the child was seven years old and in good health. No family history of hemophilia or other bleeding tendency was elicited.

Physical examination on admission showed a white woman with a small amount of continuous bleeding from gums around upper incisors, with no obvious lesions. There were scattered tiny old petechiae on the hard palate. A few fine crepitant râles in the right base largely cleared on coughing. The abdomen was protruding, with the fundus palpable at the umbilicus and fetal movements present. The liver edge was palpable 1 cm. below the right costal margin. Pelvic examination revealed slight tenderness in the bladder area and in the region of both adnexa. The findings on rectal examination were normal. Small lymph nodes were felt in the axillary and inguinal regions. The skin was slightly pale, and there were small petechiae in the left axillary fold. There were slight varicosities in the legs and a birthmark on the left knee. The blood pressure was 128/80, the temperature 97° F., and the pulse 80.

The laboratory findings were as follows:

Bleeding time, Duke,[6] over 15 min.
Lee and White clotting time, 15 min.
Hb, 11.1 Gm. per cent; *R.B.C.,* 3.34 million; *platelets,* indirect in Rees-Ecker diluent, 471,940.
W.B.C., 18,315; *diff.,* bnd. 6, seg. 82, lym. 7, mono. 5.
Prothrombin, Ware & Seegers,[7] less than 10 units per milliliter, Owren and Aas,[8] 13 per cent of normal.
Prothrombin Utilization, White, Aggeler, and Glendening,[9] less than 5 per cent.
Proconvertin, Owren and Aas,[8] less than 5 per cent of normal.
Fibrinogen, 580 mg. per cent.

During the next five days, many additional laboratory tests were conducted. The most significant findings, those relating to prothrombin and proconvertin, are summarized in Table I, together with the therapeutic regimen. However, it should be noted that the patient showed no abnormalities in thymol turbidity, cephalin flocculation, bromsulfalein, heparin titration,[10] mean corpuscular volume, mean corpuscular hemoglobin, or stool examination. Urinalysis showed specific gravity 1.015, albumin 10 mg., occasional white cells, and a few erythrocytes. Urine cultures were negative. The Dalldorf test of capillary fragility was normal and no petechiae were observed.

On the evening of admission, the patient was given 500 ml. of whole blood with 100 mg. of Mephyton, 100 mg. of protamine sulfate, and 20 mg. of Benadryl added. The bleeding stopped on the second hospital day, and on the fifth day, with the prothrombin level up

*Whitehall Pharmacal Co., acetophenetidin, sodium acetylsalicylate, and caffeine.

to 80 per cent, the patient was discharged (January 23). Subsequent to that time, she was seen in the Wadley Clinic on the dates listed in Table I. Diagnosis: bleeding due to hypoprothrombinemia and hypoproconvertinemia, cause unknown.

TABLE I. PROTHROMBIN AND PROCONVERTIN TITERS DURING MEDICATION

DATE	PROTHROMBIN* (PER CENT)	PROCONVERTIN* (PER CENT)	MEDICATION
1/18/54	13	5	500 ml. fresh, whole blood, 20 mg. Benadryl, 100 mg. protamine sulfate, 100 mg. Mephyton† (I. V.)
1/19/54	29	24	100 mg. Mephyton, 30 mg. Benadryl
1/20/54	50	39	50 mg. Mephyton, 20 mg. Benadryl
1/21/54	72	90	10 mg. menadione‡
1/22/54	81	87	10 mg. menadione‡
1/23/54	80	100	10 mg. menadione‡
1/29/54	29	14	1/24/54 to 2/4/54, 10 mg. menadione every other day
2/ 5/54	27	8	100 mg. Mephyton
2/12/54	55	69	2/6/54 to 2/12/54, 50 mg. Mephyton every other day
2/19/54	100	100	2/13/54 to 2/26/54, 100 mg. Mephyton per week
2/26/54	120	170	
3/ 5/54	100	100	2/27/54 to 3/5/54, 50 mg. Mephyton per week
3/12/54	100	200	No medication after 3/5/54
3/26/54	120	200	None
5/ 7/54	120	200	None

*Prothrombin and proconvertin expressed as per cent of normal.
†Mephyton, Merck, 2-Methyl-3-Phytyl-1, 4-Naphthoquinone, K_1.
‡Menadione, Lilly, Synthetic K_3.

On May 20, 1954, the patient delivered a normal healthy child. No unusual bleeding tendency in either mother or child was noted at that time or during the six weeks' observation period since. On July 15, 1954, the laboratory findings on Mrs. C. were as follows: prothrombin, 110 per cent of normal; proconvertin, 160 per cent of normal.

DISCUSSION

There seems to be little question that this patient was significantly deficient in vitamin K at the time of her admission. The marked depression of her prothrombin and proconvertin levels would have suggested this; her rapid and satisfactory response to Mephyton would appear to substantiate and confirm it. As noted in the case history, it is difficult to account for this avitaminosis. Despite a good and adequate dietary intake, no history of liver disease or damage, no record of drug sensitivity or poisoning, no history of Dicumarol or antibiotic therapy, and no indication of fat metabolism dysfunction, the patient presented a blood coagulation picture of hypoprothrombinemia and hypoproconvertinemia characteristic of this condition. It is of interest that this patient presented no history of a bleeding tendency prior to her admission; hence, congenital deficiency in these coagulation factors can be reasonably excluded. This view is strengthened by the fact that she has now remained at or above normal (in prothrombin and proconvertin) for a period of nineteen weeks without additional medication. Thus, a transitory, acquired hypoprothrom-

binemia and hypoproconvertinemia associated with vitamin K deficiency during pregnancy appears compatible with the facts in this case, although the etiology of this avitaminosis is obscure.

At first glance, one might conclude that the metabolic defect in this case lay in the patient's inability to assimilate and utilize oral vitamin K. Certainly, she did not respond satisfactorily to oral menadione, but rather, tended to revert to her hemorrhagic condition during such therapy. This may be more apparent than real, however, for the doses of oral menadione (5 to 10 mg. per day) were smaller than those where I. V. Mephyton was employed (7 to 100 mg. per day). This relative potency of the oil-soluble vitamin K_1 as compared with the oral, water-soluble vitamin K_3 has, of course, been noted by other investigators, and the results of medication may merely reflect the greater therapeutic effectiveness of the former material.

As has been noted by others, the proconvertin titer of this patient dropped more rapidly and to lower levels during periods of vitamin K deficiency than did her prothrombin concentration. Conversely, the proconvertin level recovered more rapidly than did the prothrombin titer when this deficiency was corrected. Thus, in this case, the proconvertin determination appeared to be a slightly more sensitive index of the patient's condition than did the prothrombin analysis. The patient's abnormal bleeding time was an unexpected and unexplained observation of interest in this case.

Although no evidence of liver damage could be found from laboratory tests, the finding of the palpable liver must be regarded with suspicion. The possibility of a very mild hepatitis or some other temporary damage of the liver sufficient to depress prothrombin and proconvertin synthesis, but not sufficient to alter the functions measured by other laboratory tests, must be considered. The patient recovered from the deficiency and did not require special vitamin therapy well before the end of pregnancy. This course seems most compatible with a period of slight dysfunction of the liver, cause unknown.

SUMMARY

A case of transient vitamin K deficiency, acquired during the fifth month of pregnancy, has been described. The hypoprothrombinemia and hypoproconvertinemia characteristic of this condition were readily corrected by intravenous Mephyton, although oral menadione, in the dosages employed, appeared ineffective. For nine weeks prior to delivery and six weeks following, there has been no recurrence of this avitaminosis, although all medication has been withdrawn. No bleeding tendency in mother or newborn child was noted at delivery. No etiology for the vitamin K deficiency of this patient has been found.

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THE BIOLOGIC DECAY RATES AND EXCRETION OF RADIOCESIUM, Cs^{134}, WITH EVALUATION AS A TRACER OF POTASSIUM IN DOGS

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AN APPROACH to the investigation of the long-term metabolic phenomena of potassium has been limited by lack of an isotope of potassium with a suitably long physical half-life. The available isotope of potassium, K^{42} ($T_{½}$ 12.44 hours), has a physical half-life which is too short to allow studies of potassium metabolism over long periods. Previous studies of *E. coli*,[1] erythrocytes,[2, 3] and other metabolic studies[4-11] suggested that Rb^{86} ($T_{½}$ 19.5 days) and Cs^{134} ($T_{½}$ 2.3 years) might be used to give at least a qualitative trace of potassium. Observations on man[12-14] and dog[15] and on erythrocytes in vitro[2, 3] have been made by use of Rb^{86}. Although the previous studies have indicated that Rb^{86} was a more reliable tracer of potassium than Cs^{134}, these studies were undertaken to determine more precisely the adequacy of Cs^{134} as a tracer of potassium in vivo. Furthermore, studies of this nature have added importance because cesium is a fission product of certain nuclear explosions.

METHODS AND MATERIALS

Three healthy mongrel dogs were placed in separate metabolic cages. For the week before the tracer was injected the dogs were placed on the same diet (Pardt†) which was employed throughout the experiment.

The dogs were anesthetized with an intravenous injection of pentobarbital sodium and a heparinized cannula was inserted into the jugular vein. Cesium^{134}, as $Cs^{134}Cl$, in a dose of 20,000,000 counts per minute per 10 pounds of body weight was injected in an average time of 5 seconds. The volume of the solution was 0.5 ml. Samples of venous blood were collected at intervals of 10 to 15 seconds immediately after injection and at increasing intervals up to 60 minutes after injection.

The animals were then returned to the metabolic cages. Urine and feces were collected each morning. The bottom pan was rinsed twice and the washings were assayed for radioactivity. Food and water remaining from the previous 24 hours were removed from the cage, measured and replaced by a known quantity of each. Blood was collected at intervals of 1 or 2 days until radioactivity reached background levels which required 45 to 47 days.

The urinary output of sodium, potassium, and chloride was determined for each 24-hour period. Sodium and potassium were determined by means of a Beckman flame photometer with suitable corrections for coexcitation[16] and chloride by the method of Schales and Schales.[17] Radioactivity of all samples was assayed by a method previously described.[18] Samples of whole blood and plasma were assayed and, utilizing the hematocrit value, the concentration of radioactivity in the erythrocytes was calculated.

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†Swift and Company.

RESULTS

1. *Rapid Rate of Exchange Between Plasma and Extraplasma Compartments.*—Fig. 1 defines the concentration-time course curves for Cs^{134} in the plasma of three dogs for the first 60 minutes following intravenous administration of the tracer. The equations, calculated by the method of Merrell and associates,[19, 20] defining these curves are shown with the respective figures.

Table I summarizes the rates of exchange of Cs^{134} between the plasma and extraplasma compartments in terms of the percentage of Cs^{134} in the respective compartments. It is evident from this table that from 0.4 to 0.8 of the Cs^{134} in the plasma was transferred to the extraplasma compartment per minute while 0.04 to 0.06 of the Cs^{134} in the extraplasma compartment was returned to the plasma per minute. Other details may be obtained by a study of Table I.

TABLE I. DISTRIBUTION AND TURNOVER OF RADIOCESIUM, RADIORUBIDIUM AND RADIOCHLORIDE IN BLOOD SERUM AND EXTRACELLULAR COMPARTMENTS OF DOGS

	Cs^{134}				Rb^{86}	Cl^{36}
	DOG NO.					
	7017	7018	7019	AVERAGE OF THREE DOGS	AVERAGE OF SEVEN DOGS	AVERAGE OF FOUR DOGS
q = Proportion of total cesium not in the serum	0.923	0.877	0.905	0.898	0.971	0.755
1 − q = Proportion of total cesium in the serum	0.077	0.123	0.095	0.102	0.029	0.245
R_s = Proportion of seral cesium leaving the serum per minute	0.769	0.439	0.407	0.552	0.811	1.02
R_e = Proportion of nonseral cesium returning to the serum per minute	0.064	0.061	0.043	0.063	0.024	0.332
$R_s \times (1 - q) = R_e(q)$ = Proportion of total body cesium moving in *or* out of the serum per minute	0.059	0.053	0.039	0.056	0.023	0.250
$R_s \times (1 - q) + R_e(q)$ = Proportion of total body cesium moving in *and* out of the serum per minute	0.118	0.106	0.078	0.112	0.046	0.500

Equations of decay curves:

Dog No. 7017	$CN_t = 28{,}000e^{-1.386t} + 28{,}000e^{-0.277t} + 4{,}700e^{-0.020t}$
Dog No. 7018	$CN_t = 23{,}000e^{-0.639t} + 14{,}800e^{-0.224t} + 6{,}350e^{-0.019t}$
Dog No. 7019	$CN_t = 31{,}500e^{-0.578t} + 13{,}700e^{-0.149t} + 4{,}750e^{-0.018t}$
Average, three dogs	$CN_t = 27{,}500e^{-0.886t} + 18{,}833e^{-0.216t} + 5{,}266e^{-0.019t}$
Rb^{86}	$CN_t = 145{,}000e^{-0.693t} + 27{,}000e^{-0.160t} + 5{,}200e^{-0.010t}$
Cl^{36}	$CN_t = 1{,}250e^{-2.079t} + 680e^{-0.308t} + 144e^{-0.038t} + 674e^{-0.0092t}$

The general configuration of the concentration-time course curves was essentially the same for the three dogs. The rate of change in concentration was about the same as that observed for Rb^{86} [15] and less than that observed for Cl^{36} [21]

TABLE II. CERTAIN OBSERVED BIOLOGIC DECAY PERIODS, IN DAYS, OF Cs^{134} IN THREE DOGS

DOG NO.	$C_{½}$			$U_{½}$	$E_{½}$
	WHOLE BLOOD	PLASMA	ERYTHROCYTES		
7017	21.5	18.5	25.5	46.0	39.0
7018	13.0	12.0	12.5	35.5	22.5
7019	16.5	12.0	18.0	27.0	17.0

(Table I). These comparisons involve small numbers of dogs, and with the individual variations which existed in such concentration-time course curves these values cannot be considered to define adequately the concentration-time course for dog.

2. *Biologic Decay Rates.*—The biologic decay rates of Cs^{134} observed for the three dogs studied are summarized in Table II and Figs. 2, 3, and 4. Initially the concentration of Cs^{134} in the erythrocytes rose while that in the plasma was declining. Thereafter, the rate of decay ($C_{½}$) for the plasma was shorter than that for either the whole blood or the erythrocytes. From 17 to 39 days were required for half of the injected tracer to be excreted by both urine and stools ($E_{½}$), whereas 27 to 46 days were required to excrete one-half by way of the urine alone ($U_{½}$).

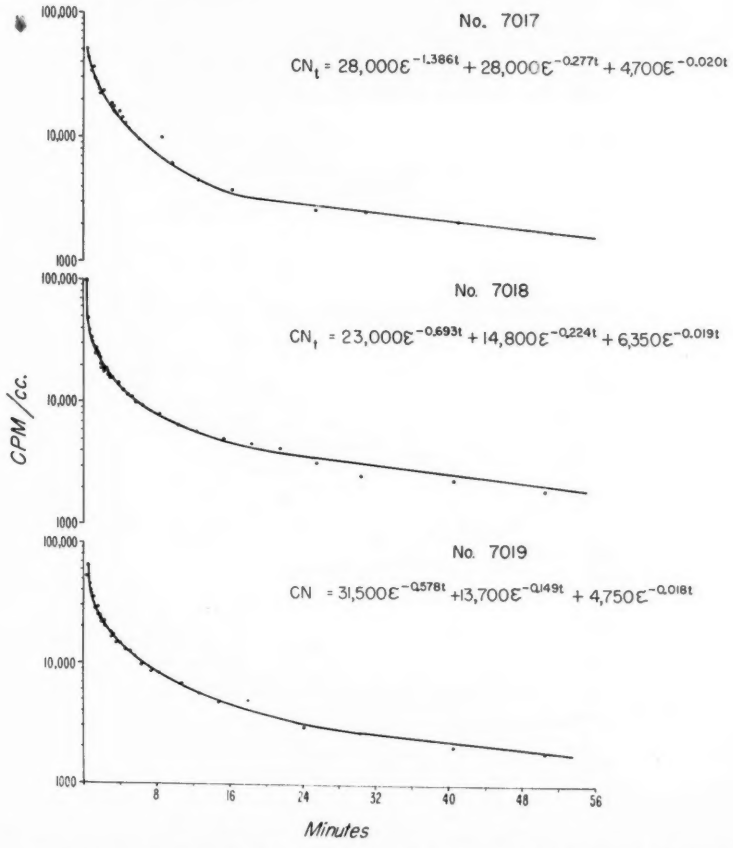

Fig. 1.—Concentration-time course of Cs^{134} in the plasma of three dogs. The relative rates of exchange are indicated by the equations shown with each regression curve.

3. *Equilibrium of Distribution.*—The difficulties in estimating the time of occurrence of equilibrium of distribution of injected isotopes have been presented previously.[12-15, 22, 23] With these difficulties in mind, an attempt was made to estimate when equilibrium of distribution had been reached for Cs^{134} in these dogs. It became evident from Figs. 2, 3, and 4 that equilibrium of dis-

tribution was never reached in these studies. During the first three to six days the concentration of Cs^{134} in the erythrocytes rose while the concentration in the plasma declined as did the percentage of the injected dose remaining in the body. During the remainder of the experiments the rate of decline in concentration in the plasma exceeded the rate of decline in the concentration in the erythrocytes and the rate of elimination from the body as a whole (Figs. 2, 3, and 4, Table II). Even had these curves manifested a uniform slope and parallelism, equilibrium of distribution would not necessarily have existed.

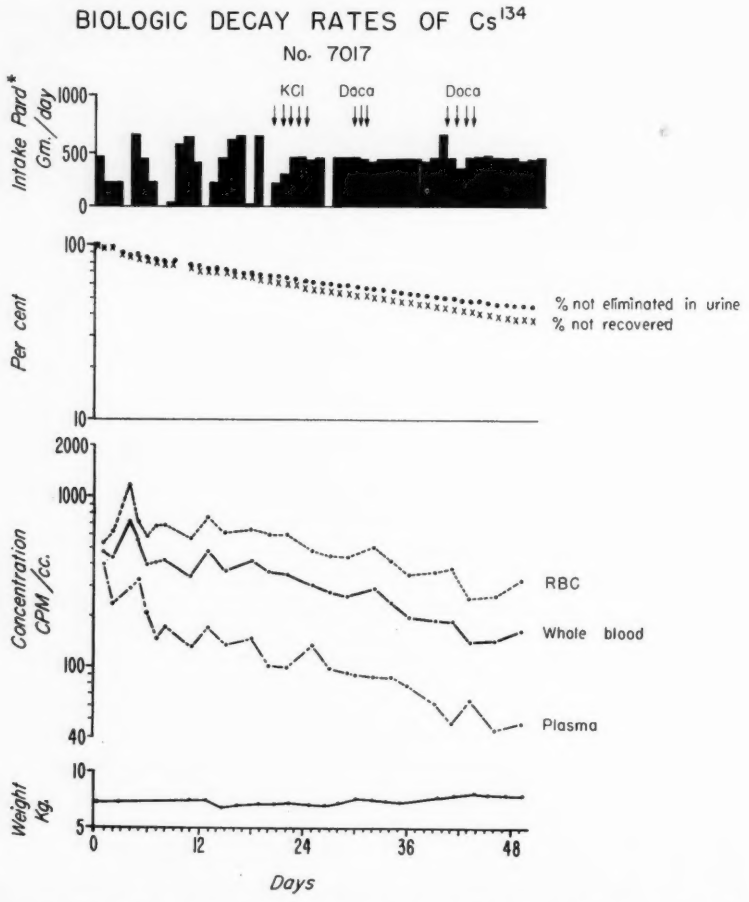

Fig. 2.—Concentration-time course of Cs^{134} in the erythrocytes, whole blood, and plasma and the time courses of urinary excretion and total excretion of Cs^{134} in Dog 7017. Urinary and total excretion are expressed as the percentage of the administered dose of tracer remaining in the body and not eliminated by the routes studied. The relationship of the dietary intake of Pard,* drug administration, and body weight is shown.

The influence of the failure to establish equilibrium of distribution on estimations of Cs^{134} space and K^{39} space and mass, even if a satisfactory trace existed, was essentially the same as that discussed for tracer studies of Rb^{86} and Cl^{36} for estimations of space and mass of K^{39} and Cl^{35}.[12, 13, 14, 24, 25] Estimations of space of Cs^{134} and of space and mass of K^{39} are discussed later.

*Swift and Company.

4. *Excretion of Cs^{134}, K^{39}, Na^{23}, and Cl^{35}.*—The urinary clearances of Cs^{134}, K^{39}, Na^{23}, and Cl^{35} are summarized in Figs. 5, 6, and 7. The dietary intake and the administration of potassium chloride and desoxycorticosterone are indicated. It is evident that although Cs^{134} did not follow the excretion of potassium qualitatively and concordantly at all times, it did follow excretion of potassium most of the time. Quantitatively the excretion of Cs^{134} and K^{39}

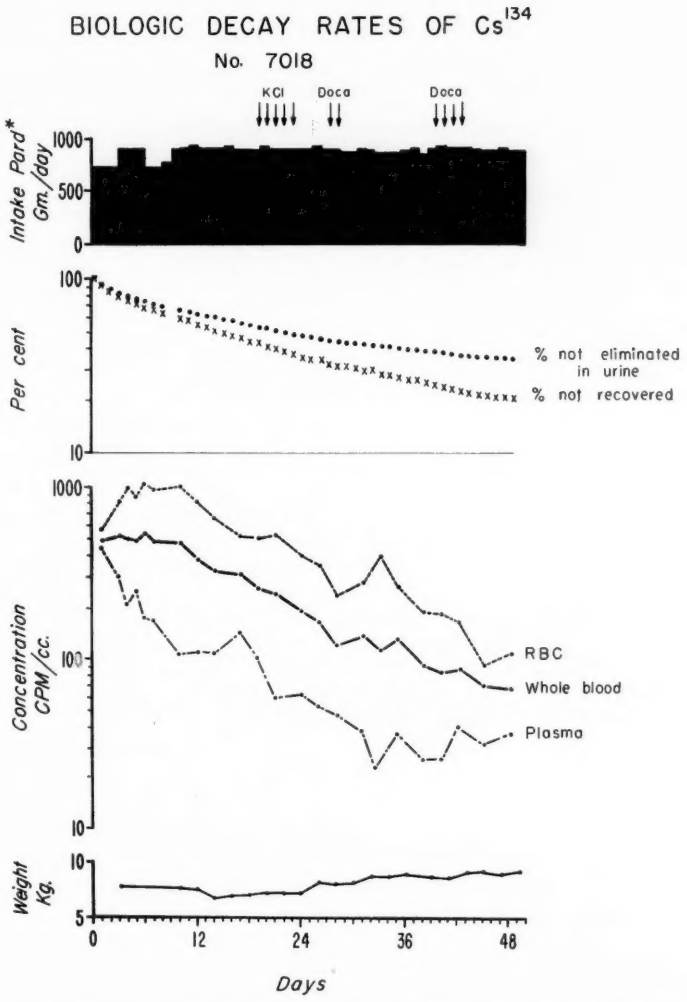

Fig. 3.—Concentration-time course of Cs^{134} in the erythrocytes, whole blood, and plasma, and the time courses of urinary and total excretion of Cs^{134} in Dog 7018.

differed markedly, the average clearances of Cs^{134} being 49.3, 43.4, and 52.0 per cent of the clearances of potassium. When extra amounts of potassium were administered orally as potassium chloride, whereas there was an increase in the clearance of potassium in all dogs, there was only a slight increase in the clearance of Cs^{134} in just one dog. On the other hand, the first time desoxycorticosterone was administered subcutaneously there was a definite decrease

in the clearance of Cs^{134} and potassium as well as sodium and chloride in all dogs. However, on the second administration of desoxycorticosterone the response was not so consistent (Figs. 5, 6, and 7). The quantity of Cs^{134} excreted by way of the urine and stools is summarized in Table III. From 55 to 80 per cent of the injected dose was recovered in the urine and from 8 to 14 per cent in the stools. The amount of Cs^{134} injected was not recovered. The difference (8 to 37 per cent) was probably due in part to sequestration of the tracer in the body.

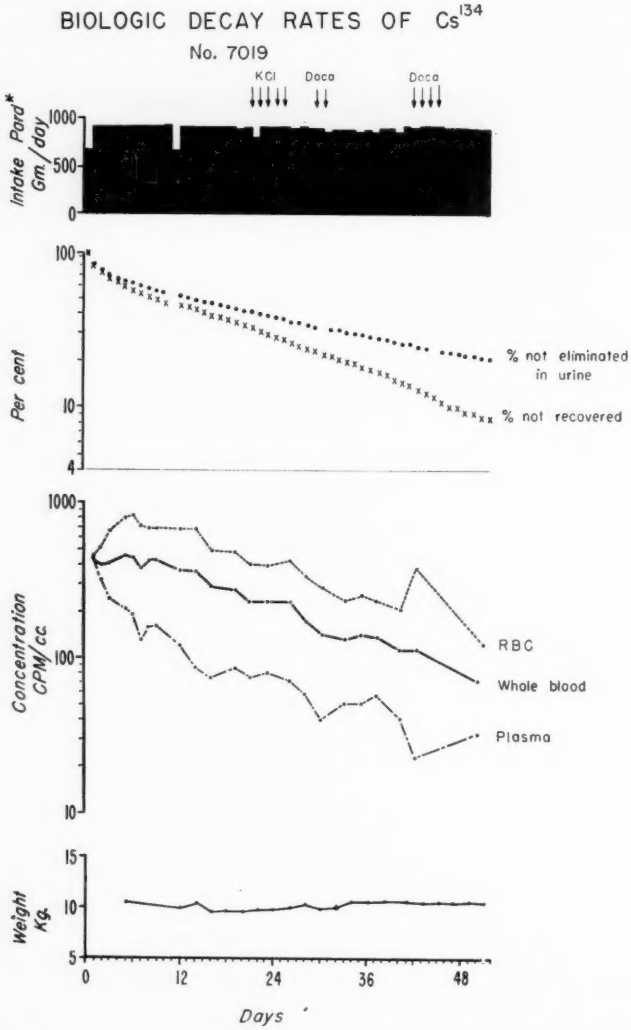

Fig. 4.—Concentration-time course of Cs^{134} in the erythrocytes, whole blood, and plasma, and the time courses of urinary and total excretion of Cs^{134} in Dog 7019.

It is interesting to note that the dogs ate 5.77, 9.22, and 7.25 per cent of their body weight in Pard per day. Assuming that dog has 55 meq. of potassium per kilogram of body weight, the dogs ingested 5.9, 10.4, and 9.0 per cent of their body potassium per day.

DISCUSSION

It is evident from the data that equilibrium of distribution was not established for Cs^{134}, that the excretory rate of Cs^{134} was only about half that of potassium, and that Cs^{134} is not a sufficiently satisfactory tracer of potassium to warrant its use for the estimation of space and mass of potassium in these dogs. As could be predicted, the use of Cs^{134} to estimate space and mass of potassium resulted in absolute values which were not sufficiently accurate to be of significance. The variations in these calculated values based on studies of the plasma compartment were considerably larger than the variations when

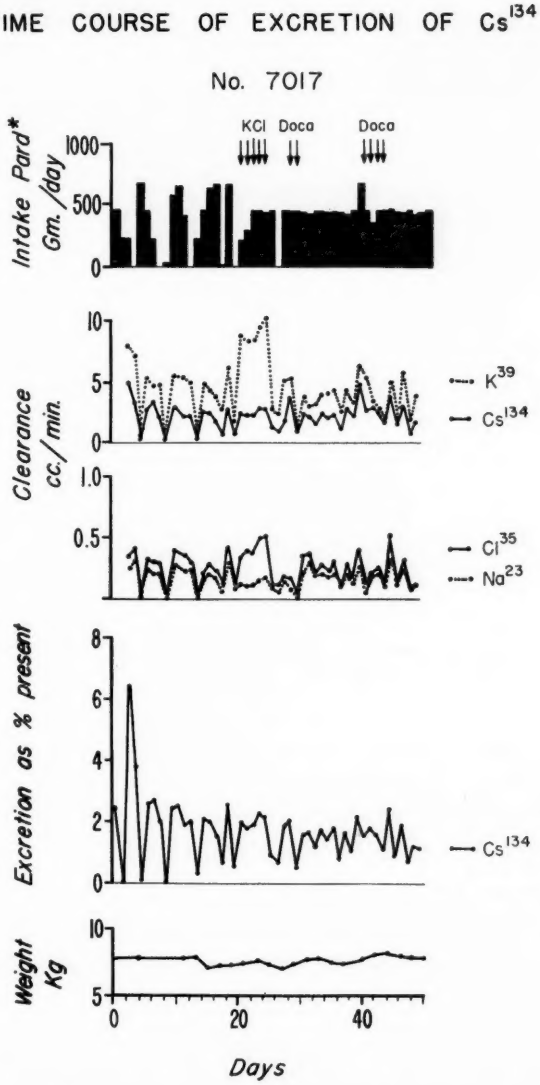

Fig. 5.—The simultaneous time-course curves of urinary clearances and of excretion of Cs^{134} by Dog 7017. The relationship to dietary intake and to various drugs is indicated.

calculations of space and mass were based on the erythrocytic compartment. This was to be expected on the basis of previous studies with Rb^{86},[13] where it was indicated that since potassium was primarily intracellular a better trace was apparent when the data were expressed in terms of an intracellular compartment of the tracer.

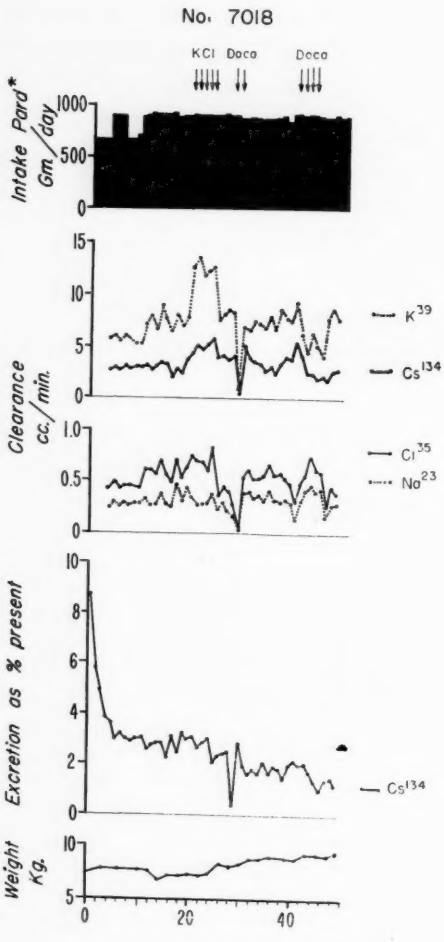

Fig. 6.

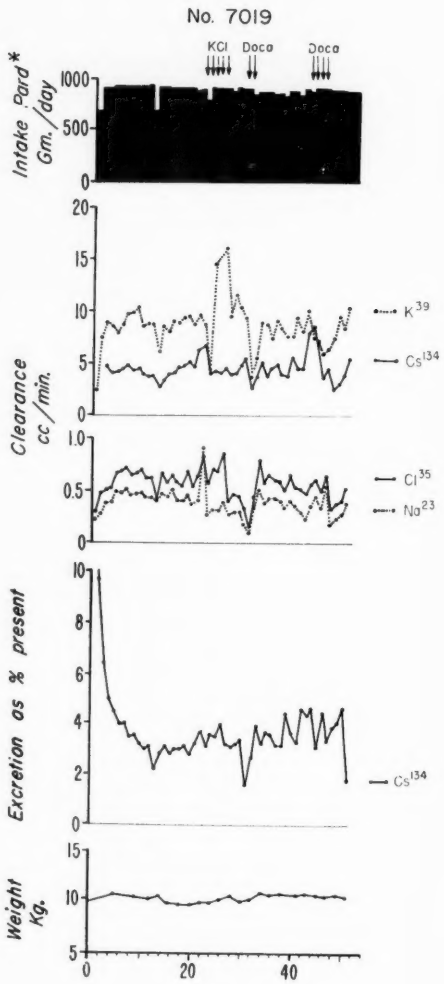

Fig. 7.

Fig. 6.—The simultaneous time-course curves of urinary clearances and of excretion of Cs^{134} by Dog 7018.

Fig. 7.—The simultaneous time-course curves of urinary clearances and of excretion of Cs^{134} by Dog 7019.

As with Rb^{86},[14] a significant lag in excretion of Cs^{134} makes unreliable the estimation of half-life or turnover rate of the element for the entire body or for any single compartment from calculations based on observations of another

TABLE III. RECOVERY OF Cs^{134} FROM THREE DOGS

DOG NO.	DURATION OF STUDY DAYS	RECOVERY AS A PERCENTAGE OF INJECTED DOSE			PERCENTAGE OF THAT RECOVERED WHICH WAS EXCRETED BY:	
		TOTAL	URINE	STOOL	URINE	STOOL
7017	49	63.0	55.0	8.0	87.3	12.7
7018	48	79.5	65.5	14.0	82.4	17.6
7019	50	91.6	79.5	12.1	86.8	13.2

compartment. This is indicated by the biologic decay rates summarized in Table II. It is safe to conjecture that these relations probably also hold for man. Their application to man contaminated with Cs^{134} is evident.

The turnover of Cs^{134} between plasma and extraplasma compartments in dogs was definitely less rapid than that of Cl^{36} but more rapid than that of Rb^{86} (Table I).[15, 21] This difference may have been due to the physical and thermodynamic differences between these elements.

Various theoretical and practical significances and applications of these experiments have been amply discussed previously for tracer studies of Cl^{36},[24, 25] Na^{22},[26, 27] and Rb^{86} [12-14]; therefore, they need not be reviewed.

SUMMARY

Radiocesium, Cs^{134}, was found to disappear from the plasma at such a rate that 0.4 to 0.8 of the tracer in the plasma was transferred to the extraplasma compartments per minute while 0.04 to 0.06 of the tracer in the extraplasma compartments returned to the plasma per minute.

The rate of disappearance of tracer from the plasma was more rapid than that from the whole blood or erythrocytes. The concentration in the erythrocytes rose during initial phases of study while the concentration in the plasma was declining.

Half of the injected tracer was excreted in stools and urine combined in 17 to 39 days, whereas 27 to 46 days were required to excrete half of the injected tracer in the urine alone. From 50 to 55 per cent of the injected dose was recovered in the urine and 8 to 14 per cent in the feces, 8 to 37 per cent remaining in the body. This was true in spite of the fact that the dogs ate from 6 to 9 per cent of their body weight in food per day. At no time was equilibrium of distribution achieved for Cs^{134} in the dogs studied for periods up to 47 days, at which time the counts in the blood reached background.

The urinary clearance rate of Cs^{134} varied from 43 to 57 per cent of the clearance rate of potassium. Apparently Cs^{134} is not a satisfactory tracer of potassium, certainly not as satisfactory as Rb^{86}.

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LABORATORY METHODS

A RAPID AND SIMPLE METHOD FOR CONDUCTING LARGE SERIES OF COMPLEMENT FIXATION TESTS AND ANTISTREPTOLYSIN "O" TITRATIONS

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INTRODUCTION

THE number of complement fixation tests for syphilis is increasing steadily in many laboratories. One of the causes is the increasing demand for the services of blood transfusion donors whose blood has to be tested regularly. Another cause is the fact that more attention is being paid to preventive medical care, for instance, of gravidae. In conventional methods, test tubes are used for these tests. In order to reduce the large number of tubes required for the titrations a simple modification has been devised. Complement fixation tests are carried out not in tubes, but in Perspex sheets provided with cylindrical flat-bottomed cups. The method may be applied in any reaction that has to be read by observing the degree of hemolysis. These sheets have been employed by us also for antistreptolysin "O" titrations.

A description is given of the methods for performing complement fixation tests and antistreptolysin "O" determinations in Perspex sheets.

MATERIALS

The sheets are made from Perspex, a transparent plastic. The dimensions are 200 mm. square and 20 mm. thick. Twelve rows of twelve cups are bored in one sheet. The cups have a diameter of 12.5 mm. and a depth of 18 mm. The bottom of each cup is flat, polished, and completely transparent. The four outer sides of the bottom of the sheets are provided with thin strips of Perspex in order to prevent scratches on the underside of the sheet. A Perspex sheet with a thickness of 2.4 mm. is used as cover during the tests. The cups may be numbered with a glass pencil.

Immediately after use the sheets are rinsed in running tap water and then placed in 1 per cent hydrochloric acid for one night. Afterwards they are rinsed thoroughly in running tap water and finally in distilled water. The sheets are allowed to dry at room temperature or at 37° C. In this way the whole procedure for cleaning one sheet may be accomplished within a few minutes.

A simple metal rack is constructed to hold the sheet in the water bath at 37° C. Four horizontal strips of metal are supported by four legs. One sheet may be placed upon the rack and is held in position by small vertical strips of metal along the outer sides of the frame. The bottom surface of the sheet should be 0.5 cm. under the water level.

Volumes of 0.25 ml. of the reagents are pipetted into the cups with an automatic pipette (Type Cornwall) of 2 ml. (Fig. 1). The pipette is provided with a small, short delivery needle (No. 23). In this way volumes of 0.25 ml. may be transferred with a standard deviation of 1 per cent. Serial twofold dilutions may be prepared immediately in the cups with the aid of a syringe. A tuberculin syringe of 1 ml. is so modified that quantities of exactly 0.25 ml. are delivered. The external part of the plunger is provided

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with a spring. The fluid is drawn up in the syringe through a small, short needle by the action of the spring and forced out of the syringe by pressing on the piston. A volume of 0.25 ml. of the fluid to be diluted is mixed in a cup with an equal amount of saline solution and transferred to another cup.

A strong light with a small aperture (diameter, 5 mm.) is employed for reading the tests. The sheets are kept above the light at a distance of about 10 cm. (Fig. 2). In this way the light that passes through the fluid from below is seen separately in each cup either as a concentrated point or as a partly or completely diffuse spot in accordance with the degree of hemolysis.

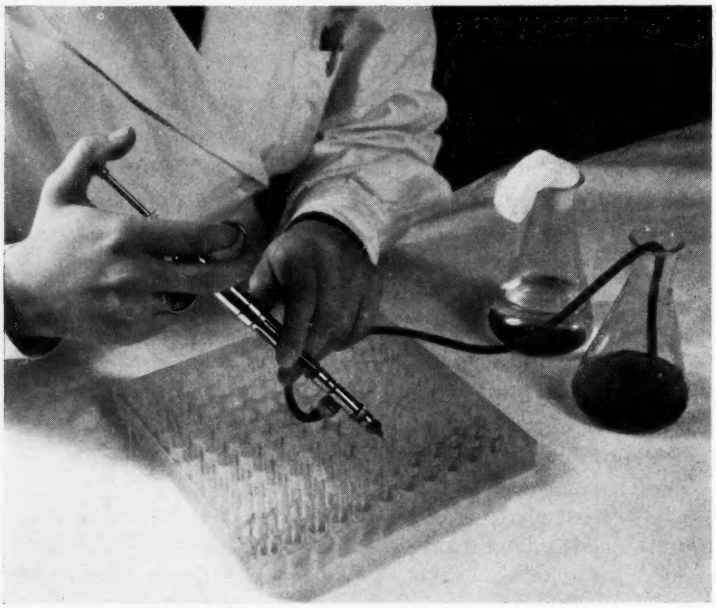

Fig. 1.—Pipetting of reagents into cups of a Perspex sheet.

METHODS

Reading Scale.—Until experience has been gained in the reading of reactions, it is advisable to prepare a scale of hemolysis according to the method employed by Kolmer and Boerner.[1] A hemoglobin solution is made by mixing 2 ml. of a 10 per cent suspension of washed sheep cells with 2 ml. of distilled water. After complete hemolysis has occurred, 0.4 ml. of the hemoglobin solution is diluted with 5.6 ml. of saline solution. A diluted suspension of sheep cells is prepared by adding 5 ml. of saline solution to 1 ml. of a 2 per cent suspension of sheep cells.

The following amounts of the diluted suspension of sheep cells are transferred to a series of six cups of a Perspex sheet: 0.75 ml., 0.36 ml., 0.18 ml., 0.09 ml., and 0.04 ml.; Cup 6 receives no sheep cells. The following amounts of the diluted hemoglobin solution are added successively to Cup 2 through Cup 6, inclusive: 0.36 ml., 0.57 ml., 0.66 ml., 0.71 ml., and 0.75 ml.; Cup 1 receives no hemoglobin solution.

The contents of the cups are mixed by rotating the sheet. The differences in the reading of the degree of hemolysis are distinct. The results from Cup 1 to Cup 6, inclusive, are recorded as: 4+, 3+, 2+, 1+, ±, and negative (Fig. 3).

Wassermann Test.—The technique is the same as in the Kolmer complement fixation test for syphilis[1] except in the modification of some minor details. The unit of volume for all reagents in the test is 0.125 ml. However, two volumes of antigen (0.25 ml.) are used. In order to obtain a dose of antigen equivalent to the dose employed in the Kolmer

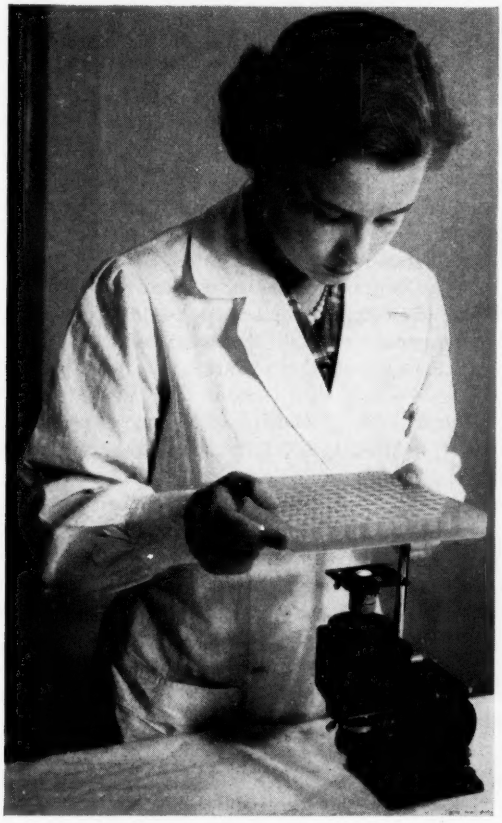

Fig. 2.—Reading of tests performed in a Perspex sheet.

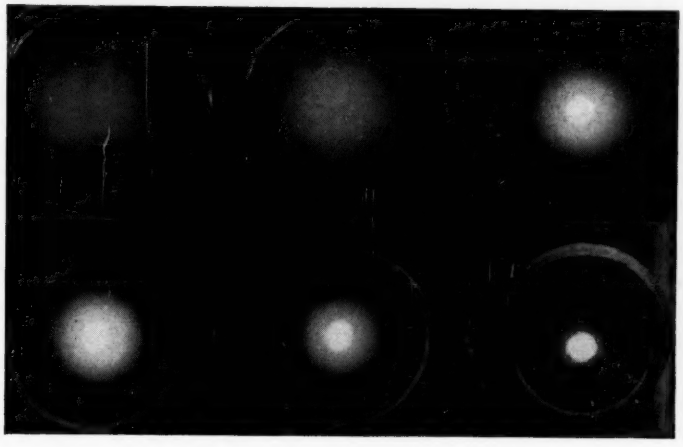

Fig. 3.—Scale of hemolysis prepared in a Perspex sheet according to the Kolmer method Results from Cup 1 to Cup 6 inclusive: 4+, 3+, 2+, 1+, ±, and negative.

complement fixation test, the antigen is diluted two times more than listed on the antigen bottle label or than determined by the Kolmer antigen titration in tubes. In accordance with the Kolmer test, two volumes of complement containing two full units and two volumes of hemolytic system are employed.

Titration of hemolysin: The following dilutions of hemolysin are prepared in tubes: 1:1,000, 1:2,000, 1:3,000, 1:4,000, 1:5,000, 1:6,000, 1:8,000, and 1:10,000. Amounts of 0.25 ml. of each dilution are transferred to the cups of a Perspex sheet. To each cup is added 0.25 ml. of 1:50 diluted complement and 0.25 ml. of a 1 per cent suspension of sheep cells. The sheet is rotated and incubated in a water bath of 37° C. for one hour. The contents of the cups are mixed again and the titration is read. No account is made for the final dilutions of hemolysin. The highest dilution of hemolysin showing complete sparkling hemolysis is recorded. For the complement titration and for the complement fixation test, hemolysin is diluted four times less than the recorded titer. The hemolytic system is prepared by mixing equal parts of this dilution of hemolysin and of a 2 per cent suspension of sheep cells.

Titration of complement: The following dilutions of complement are prepared in tubes: 1:54, 1:60, 1:66, 1:74, 1:86, 1:100, 1:120, and 1:150. Amounts of 0.25 ml. of each dilution are transferred to the cups of a Perspex sheet. To each cup is added 0.25 ml. of diluted antigen. Antigen is diluted two times more than listed on the antigen bottle label or than determined by the Kolmer antigen titration in tubes. After addition of antigen the sheet is rotated and incubated in a water bath of 37° C. for one hour. Finally, 0.25 ml. of hemolytic system is pipetted into each cup. The hemolytic system is prepared as indicated by the titration of hemolysin. After addition of the hemolytic system the sheet is rotated and returned to the water bath of 37° C. for one hour. The contents of the cups are mixed again and the titration is read. No account is made for the final dilutions of complement. The dilution of complement containing one full unit of complement is recorded. For the complement fixation test, complement is diluted two times less than the recorded titer.

Regular simplified complement fixation test: The dilution of complement to be used is indicated by the complement titration. Dilutions of antigen and hemolytic system are prepared in the same way as in the complement titration.

Three cups are employed for each serum. The first row of cups are numbered to correspond to the sera to be tested. Amounts of 0.05 ml. of undiluted serum are transferred to the first and second cup and 0.025 ml. to the third cup. To the first cup of each serum is added 0.25 ml. of saline solution. This cup serves as serum control. To the second and third cup of each serum is added 0.25 ml. of antigen dilution. The sheets are allowed to stand at room temperature for 5 to 15 minutes. To all cups is added 0.25 ml. of diluted complement. The contents of the cups are mixed by rotating the sheets. The sheets are covered and placed at 2° to 4° C. for 16 to 18 hours. Afterwards they are incubated in a water bath of 37° C. for 10 to 15 minutes. After removal from the water bath, 0.25 ml. of hemolytic system is added to all cups. The contents of the cups are mixed before returning the sheets to the water bath of 37° C. After 25 to 30 minutes the sheets are rotated and the tests are read. The results are recorded in the same way as in the Kolmer complement fixation test. The tests are completed with antigen, hemolytic system, and cell controls. A quantitative test with a known positive serum is included also as control.

Regular quantitative complement fixation test: Two dilutions of serum are prepared in test tubes as follows: 1:2 and 1:8. The following amounts of serum are transferred to six cups of a Perspex sheet:

0.05 ml. of undiluted serum to Cup 1,
0.05 ml. of undiluted serum to Cup 2,
0.05 ml. of 1:2 diluted serum to Cup 3,
0.025 ml. of 1:2 diluted serum to Cup 4,
0.05 ml. of 1:8 diluted serum to Cup 5, and
0.025 ml. of 1:8 diluted serum to Cup 6.

Cup 1 serves as serum control. Actually, 0.125 ml. of diluted serum should have been transferred to each cup because the unit of volume for all reagents in the test described is 0.125 ml. If account is made for this volume, the following dilutions of serum are present in Cup 2 to Cup 6, inclusive: 1:2.5, 1:5, 1:10, 1:20, and 1:40. These dilutions—not the final serum dilutions—are recorded at the end of the test.

The quantitative test is completed in the same way as in the regular simplified test.

Complement Fixation Test for Bacterial and Viral Diseases.—In order to determine the optimal dose of antigen to be used for the test, a known positive serum is tested with various dilutions of antigen. The test is performed in the same way as in the quantitative Wassermann test. However, two exact units of complement are employed instead of two full units. Each serum dilution is tested with a series of twofold dilutions of antigen. The dilution of antigen showing a 4+ reaction with the smallest amount of serum is recorded. This dilution of antigen is employed in the complement fixation test.

The titration of complement and the regular simplified or quantitative complement fixation test are conducted in exactly the same way as in the Wassermann test, except that two exact units of complement are employed.

Antistreptolysin "0" Titrations (AST "0").—A modification of the technique of Kalbak[2] has been employed. In accordance with the Kalbak method the number of AS "0" units in the patient's sera are calculated on the basis of the results of a standardized serum titrated simultaneously. However, the volumes employed for the tests, the range of serum dilutions, and the final concentration of rabbit cells are chosen differently than in the Kalbak method.

Titration of streptolysin "0": Two series of twofold dilutions of streptolysin "0" are prepared in the cups of a Perspex sheet as follows: 1:6, 1:8, 1:12, 1:16, 1:24, 1:32, 1:48, 1:64, 1:96, 1:128, 1:192, and 1:256. The dilutions are made in saline solution. To each cup is added 0.25 ml. of a 1 per cent suspension of rabbit cells and 0.25 ml. of saline solution. The sheet is rotated and incubated in a water bath of 37° C. for 45 minutes. The contents of the cups are mixed again and the titration is read. No account is made for the final dilutions of streptolysin "0." The highest dilution of streptolysin "0" showing complete sparkling hemolysis is recorded. This dilution contains one minimum hemolytic dose (1 MHD). For the standardization of streptolysin "0," amounts of 0.25 ml. of streptolysin "0" containing 2, 2½, and 3 MHD are employed.

Standardization of streptolysin "0": A standardized serum containing 10 units of antistreptolysin "0" (AS "0") is employed. A series of dilutions of standardized serum are prepared in tubes containing the following numbers of AS "0" units: 1.56, 1.25, 1.00, 0.80, 0.64, 0.50, 0.40, and 0.32. These dilutions may be made by transferring successively four parts of diluted serum to one part of saline solution, starting with a dilution containing 1.56 AS "0" units. Amounts of 0.25 ml. of each dilution are transferred to the cups of a Perspex sheet. Three series of serum dilutions are prepared. To the first series is added 0.25 ml. of streptolysin "0" containing 2 MHD, to the second series 0.25 ml. of streptolysin "0" containing 2½ MHD, to the third series 0.25 ml. of streptolysin "0" containing 3 MHD. The sheet is rotated and incubated in a water bath of 37° C. for 15 minutes. Finally, 0.25 ml. of a 1 per cent suspension of rabbit cells is pipetted into each cup. The sheet is returned to the water bath for 45 minutes. The contents of the cups are mixed again and the titration is read. The series presenting complete inhibition of hemolysis in the first cups and sparkling hemolysis in the last cups, with only a few cups showing partial hemolysis, contains the optimal number of MHD for the test described. For the titration of the patient's sera, 0.25 ml. of streptolysin "0" containing the optimal number of MHD is employed.

Antistreptolysin "0" titration of patient's sera: Two series of two-fold dilutions of serum are prepared in the cups of a Perspex sheet as follows: 1:50, 1:75, 1:100, 1:150, 1:200, 1:300, 1:400, 1:600, 1:800, 1:1,200, 1:1,600, and 1:2,400. Streptolysin "0" is diluted as indicated by the standardization of streptolysin "0." After addition of 0.25 ml. of diluted streptolysin "0" the sheet is rotated and incubated in a water bath of 37° C. for 15 minutes. Finally, 0.25 ml. of a 1 per cent suspension of rabbit cells is pipetted into each cup. The sheet is returned to the water bath for 45 minutes. The contents of the

cups are mixed again and the titration is read. The highest serum dilution showing a 4+ reaction is recorded. A titration of a standardized serum containing the same amounts of AS "0" units as in the standardization of streptolysin "0" is included. The number of AS "0" units present in the highest dilution of standardized serum showing a 4+ reaction is recorded. The number of AS "0" units present in the patient's sera is calculated by multiplying the recorded titer with the recorded number of AS "0" units of the standardized serum. The number of AS "0" units are rounded out as indicated by Table I. The tests are completed with streptolysin "0" and cell controls. Two known positive sera are included also as controls.

TABLE I. CALCULATED NUMBER OF AS "0" UNITS PRESENT IN PATIENT'S SERA

AS "0" UNITS OF STANDARDIZED SERUM*	DILUTION OF PATIENT'S SERA†											
	1:50	1:75	1:100	1:150	1:200	1:300	1:400	1:600	1:800	1:1,200	1:1,600	1:2,400
1.56	75	100	150	200	300	400	600	800	1,200	1,600	2,400	3,200
1.25 1.00 0.80	50	75	100	150	200	300	400	600	800	1,200	1,600	2,400
0.64 0.50 0.40	25	37	50	75	100	150	200	300	400	600	800	1,200

*Number of AS "0" units present in highest dilution of standardized serum showing a 4+ reaction. Generally, streptolysin "0" is so standardized that the highest dilution of standardized serum showing a 4+ reaction contains 1.00 AS "0" unit.

†Highest serum dilution showing a 4+ reaction.

RESULTS

In view of the differences in shape and in material between the Perspex sheets described and test tubes ordinarily employed, some factors that consequently might influence the results of the tests have been investigated.

If the sheets are covered with the thin Perspex sheets during the incubation periods in the icebox or in the water bath of 37° C., small droplets distill over on to the cover sheets just above the cups. One may assume that the volume of these droplets is equivalent to $\frac{1}{3} \pi r^3$, r being the radius of a droplet. After measuring the diameter of the droplets and counting the number of droplets, the amount of water distilled over on to the cover sheet may be calculated with the aid of this formula. It was found that the amount of fluid evaporated after completion of Wassermann tests was less than 1 per cent of the total volume. Measurements obtained by weighing sheets before and after completion of Wassermann tests have shown also that less than 1 per cent of the volume evaporates.

Amounts of 0.75 ml. of saline solution were pipetted into various cups of a Perspex sheet and into a series of test tubes (75- by 10-mm. inside diameter). The sheet and test tubes were incubated in a water bath of 37° C. for a period of 30 minutes. The temperature of the water present in the cups and in the test tubes was measured at various intervals. It was found that the maximum temperature was obtained in the sheet about 5 minutes later than in the test tubes. Therefore, in complement fixation tests and in AS "0" titrations the sheets are incubated for 5 minutes longer than as indicated in the methods for test tubes.

The accuracy of the technique was tested by simultaneously performing a series of twenty quantitative Wassermann tests with one known positive serum. Identical titers were obtained in all tests; in two serum samples, differences of 1 and 2+ were seen in the highest dilution with inhibition of hemolysis. Moreover, AS "0" titrations were performed simultaneously with twenty samples of one known positive serum. Variation in AS "0" titer occurred once.

The Wassermann titers of a series of twenty positive sera tested in sheets were compared with the titers obtained with the Kolmer complement fixation test in tubes. The same antigen, complement, and hemolytic system was employed in both tests. Identical titers were found except in one serum sample. No significant differences were seen between the AS "0" titers of sera tested in sheets and the AS "0" titers of the same sera obtained with the Kalbak method in tubes.

DISCUSSION

A complement fixation test on Perspex sheets has been designed by Fulton and Dumbell.[3] However, their method differs in various respects from the one described in this paper. The reactions are carried out not in cups, but in drops on Perspex sheets. Consequently, the tests may be performed with smaller amounts of material than by the cup method. The titrations are read not by measuring the degree of hemolysis, but by observing the density of the dots of unhemolyzed cells. The method of Fulton and Dumbell has some disadvantages: (a) The accuracy of the test is decreased by the use of small volumes delivered by dropping pipettes and by the method of reading the tests. (b) Special precautions must be taken to avoid too much evaporation during the procedure. (c) Collection of unhemolyzed cells in a dot is presumed to occur because the hemolysin dilution for maximal sensitization is close to the agglutination titer of the hemolysin serum. Therefore, it is improbable that AS "0" titrations may be performed in drops on sheets. (d) It is impossible to prepare serial dilutions of serum immediately on the sheets.

In our opinion the method described in this paper offers some distinct advantages over other conventional methods for complement fixation tests and AS "0" titrations employing tubes: (a) Less time is consumed for pipetting reagents into the sheets and for cleaning the sheets. (b) Less space is required for sheets than for an equivalent number of racks with test tubes. (c) The tests may be read rapidly and distinctly.

The technique is accurate and reproducible. The titers of complement fixing antibodies and of AS "0" antibodies found in sheets may be compared immediately with the titers obtained in test tubes. After an experience of three years no deterioration of the quality of the sheets has been observed. The sheets remain perfectly transparent.

SUMMARY

A method for performing complement fixation tests and antistreptolysin "0" titrations in transparent plastic sheets is described. The sheets are provided

with cylindrical flat-bottomed cups. Details of the method are presented. The complement fixation test is carried out in accordance with the Kolmer technique except in the varying of some minor details. A modification of the AS "0" titration described by Kalbak is applied.

The use of sheets saves a considerable amount of space and time not associated with a loss of accuracy. The titers of positive sera found with the aid of sheets are as equally high as those obtained with the aid of test tubes.

REFERENCES

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2. Kalbak, K.: The Antistreptolysin Reaction (A.S.R.) Technic, Copenhagen, 1947.
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Erratum

In the article by S. H. Armstrong, Jr., and associates, entitled "The Persistence in the Blood of the Radioactive Label of Albumins, Gamma Globulins, and Globulins of Intermediate Mobility. II. Comparison of Gamma Globulins Labeled With S^{35} and I^{131} in the Same Subjects," which appeared in the January, 1955, issue of the JOURNAL, pages 51-60, the third line on page 52 should read as follows: "labeled preparation to the gamma fraction in the same human subjects."